SYNTHESES AND METABOLIC PROFILES OF AMINOPOLYAMINES IN THE MURINE LEUKEMIA L1210 CELL AND THE PATHOGENIC BACTERIUM *Pseudomonas aeruginosa*

By

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To my wonderful grandmother, my parents, and my beautiful fiancé Christina Ngoc Cao.
To my chemistry teacher Đang Buom, who is always in loving memory.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
AcOH	acetic acid
ADC	arginine decarboxylase
AdoMetDC	S-adenosylmethionine decarboxylase
ATP	adenosine triphosphate
C	carbon
$CHCl_3$	chloroform
CNS	central nervous system
DAP	1,3-diaminopropane
DCHA	dicyclohexyl ammonium sulfate
DEHSPM	diethylhomospermine
DENSPM	diethylnorspermine
DFMA	difluoromethylarginine
DFMO	difluoromethylornithine
DMF	dimethylformamide
DNA	deoxyribonucleic acid
e.g.	for example
eIF-5A	eukaryotic translation initiation factor 5A
EtOAc	ethyl acetate
EtOH	ethanol

Expt	experiment
FAD	flavin adenine dinucleotide
HBr	hydrobromic acid
HCl	hydrochloric acid
HEPES	*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
Hex	hexane
HSPD	homospermidine
KOAc	potassium acetate
MDL 72527	*N*,*N*'-bis(2,3-butadienyl)-1,4-butanediamine
MFMO	monofluoromethylornithine
MGBG	methylglyoxylbis(guanylhydrazone)
MOPS	3-[*N*-morpholino]propanesulfonic acid
Mts	mesitylenesulfonyl
N	nitrogen
7-NH_2-DESPD	N^1,N^8-diethyl-7-aminospermidine
6-NH_2-DESPM	N^1,N^{12}-diethyl-6-aminospermine
2-NH_2-PUT	2-aminoputrescine
7-NH_2-SPD	7-aminospermidine
6-NH_2-SPM	6-aminospermine
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
2-OH-PUT	2-hydroxyputrescine

6-OH-SPD	6-hydroxyspermidine
7-OH-SPD	7-hydroxyspermidine
PAO	polyamine oxidase
Pd/C	palladium on activated carbon
PUT	putrescine or 1,4-diaminobutane
dcSAM	decarboxylated *S*-adenosylmethionine
SAM	*S*-adenosylmethionine
SPD	spermidine
SPM	spermine
SSAT	spermidine/spermine N^1-acetyltransferase
THF	tetrahydrofuran
TLC	thin-layer chromatography
t-RNA	transfer ribonucleic acid

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SYNTHESES AND METABOLIC PROFILES OF AMINOPOLYAMINES IN THE MURINE LEUKEMIA L1210 CELL AND THE PATHOGENIC BACTERIUM *PSEUDOMONAS AERUGINOSA*

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The aim of our study was to extend our understanding of polyamine metabolism by investigating how eukaryotes and prokaryotes metabolically process exogenous polyamines. A new series of polyamines, the aminopolyamines, were first assembled. Their metabolic profiles and impact on cellular and bacterial growth were measured. These newly synthesized aminopolyamines were screened in both eukaryotic and prokaryotic models. In the eukaryotic model, the murine leukemia L1210 cells were exposed to each aminopolyamine, and the 48-h and 96-h IC_{50} values were measured (in addition to their effect on polyamine metabolism). In the prokaryotic model, *P. aeruginosa* was used to study the impact of the synthetic aminopolyamines on its growth and polyamine metabolism.

Studies in L1210 cells revealed that 2-aminoputrescine (2-NH_2-PUT), and 6-aminospermine (6-NH_2-SPM) had no significant impact on cellular growth with 48-h IC_{50} values > 100 μM; while 7-aminospermidine (7-NH_2-SPD) had a very moderate effect

with a 48-h IC_{50} of 60 μM and a 96-h IC_{50} of 9 μM. The aminopolyamine N^1,N^8-diethyl-7-aminospermidine (7-NH_2-DESPD) also showed a mild effect on cellular growth with a 48-h IC_{50} of 100 μM and a 96-h IC_{50} of 6 μM. At a concentration of 1 μM, 7-NH_2-SPD and 7-NH_2-DESPD had a slight impact on ODC by reducing the enzymatic activity to 72% and 79% of control, respectively. The aminopolyamine 6-NH_2-SPM was more active by reducing ODC activity to 52% of control. In addition, 6-NH_2-SPM reduced AdoMetDC activity to 70% of control. While 7-NH_2-SPD and 6-NH_2-SPM did not significantly stimulate spermidine/spermine-N^1-acetyltransferase (SSAT), 7-NH_2-DESPD showed a moderate SSAT stimulation by upregulating SSAT to 303% of control. L1210 cells were able to catabolically convert 7-NH_2-SPD to 2-NH_2-PUT and biosynthesize 6-NH_2-SPM from 7-NH_2-SPD. Besides, when exposed to 6-NH_2-SPM, these cells converted this aminopolyamine to 7-NH_2-SPD, and then to 2-NH_2-PUT. When exposed to 7-NH_2-DESPD or 6-NH_2-DESPM, deethylation of these diethylated aminopolyamines also occurred inside the L1210 cell, possibly via the SSAT/PAO pathway.

In *P. aeruginosa*, 2-OH-PUT and 7-OH-SPD are not produced endogenously. However, when exposed to 2-OH-PUT, the bacteria elaborated 2-OH-PUT into 7-OH-SPD. A similar scenario occurred in which 2-NH_2-PUT was converted to 7-NH_2-SPD by *P. aeruginosa*. This bacterium was incapable of producing 6-NH_2-SPM from 7-NH_2-SPD. However, when exposed to 6-NH_2-SPM, it was able to metabolize 6-NH_2-SPM to 7-NH_2-SPD and 2-NH_2-PUT. Our results in this dissertation significantly add to the understanding of how various polyamine analogues are metabolically processed in both eukaryotes and prokaryotes.

CHAPTER 1
INTRODUCTION

Polyamines are essential for the growth and development of prokaryotes and eukaryotes. Life without polyamines is not known, with the exception of a few microorganisms, such as those in the order Halobacteriales. Many research studies, including those in our laboratory (Bergeron, 1994, 1995a, 1995b, 1997, 1999, 2000, 2001a, 2001b), show that disruption of the polyamine metabolic pathway is a promising target for anticancer and antibacterial strategies. Polyamine analogues that can block biosyntheses or interfere with the function of naturally occurring polyamines have considerable potential as therapeutic agents. Insights into polyamine metabolism are important in understanding how these drugs work. This chapter focuses on the metabolism of polyamines in prokaryotes and eukaryotes.

Polyamines and their roles

Polyamines are small aliphatic molecules containing two or more amino moieties separated by carbon chains. For instance, spermidine (SPD) is a triamine in which the three amino groups are separated by a group of three methylenes and a group of four methylenes (Fig. 1-1). It is designated as a (3-4) triamine. Similarly, putrescine (PUT) is denoted as a (4) diamine, and spermine (SPM) as a (3-4-3) tetraamine. Thus, the number of methylene groups is used to denote various polyamine structures.

The principal naturally occurring polyamines in eukaryotes are PUT, SPD, and SPM (Fig. 1-1); although 1,3-diaminopropane (DAP) and hydroxypolyamines (including 2-hydroxyputrescine (2-OH-PUT) and 7-hydroxyspermidine (7-OH-SPD)) also exist in

some prokaryotes. Polyamines are essential for the growth and development of both eukaryotes and prokaryotes. While the three most common polyamines (PUT, SPD and SPM) have been found in eukaryotes, SPM is less common in prokaryotes. In some eukaryotes and prokaryotes, cationic polyamines interact with anionic macromolecules such as nucleic acids, phospholipids, and certain proteins (Igarashi and Kashiwagi, 2000). However, understanding the precise roles of polyamines in cellular physiology is still a challenge (Casero and Woster, 2001). Without polyamines, cell growth is not observed, with the exception of two bacterial orders: Methobacteriales and Halobacteriales (Hamana and Matsuzaki, 1992, 1993). Studies show that cellular and bacterial growths are slowed by the inhibition of polyamine biosyntheses (Bergeron, 2000 and Bitonti, 1982). Because of the positively charged nature at physiological pH, polyamines are believed to be involved in stabilizing DNA and initiating DNA synthesis (Matthew, 1993). The DNA undergoes condensation, conformational changes, aggregation and resolubilization in the presence of positively charged polyamines (Vijayanathan, 2001). *In vitro*, calf thymus DNA formed a diffused, planar structure, with entrapped bubbles; but when exposed to SPD and SPM, it assumed a characteristic fingerprint structure with hexagonal order (Saminathan, 2002). Polyamines are also involved in RNA synthesis and metabolism (Morgan, 1999). For instance, SPM was shown to stabilize the conformation of the anticodon loop yeast t-RNA (Tabor and Tabor, 1984; Cohen and McCormick, 1979). Polyamines are involved in stimulating RNA processing through the spermidine-regulated protein, eIF-5A, and also in stimulating Ile-tRNA formation (Igarashi and Kashiwagi, 2000). Polyamines also play an important role in protein biosynthesis,

including both initiation (Yoshida, 2001) and elongation processes (Scalabrino and Ferioli, 1982).

Roles of Polyamines in DNA Stabilization and Interactions

In the early 1960s, Tabor discovered that polyamines could stabilize double-stranded DNA (Tabor, 1962). For instance, PUT, SPD and SPM can increase the melting temperature of DNA *in vitro* by as much as 40 °C. In addition to electrostatic interaction with DNA, polyamines interact with nucleic-acid bases, and enter the minor or major grooves (Feuerstein, 1991). Polyamines, present in eukaryotic cells at millimolar concentration, are believed to have significant roles in stabilizing DNA structure. Observed accumulation of polyamines on the chromosomes of mitotic cells further indicates that polyamines have important interactions with DNA.

Polyamines in Cellular Growth and Development

Polyamines are required during each phase of the cell cycle. The cell cycle generally consists of four phases: G1, the first growth phase; S, the DNA synthetic phase; G2, the second growth phase; and M, the mitotic phase. Increased polyamine levels have been observed during cell-cycle progression. Early studies indicate elevated levels of polyamines in G1 and S phases (Heby, 1973). These studies also showed that depletion of polyamines inhibited the cellular transition from G1 phase to S phase. In Chinese hamster ovary cells, spermidine was increased during the entire cell cycle, while putrescine was increased during S and G2 phases, and spermine was increased mainly during G1 and S phase. Inhibition of polyamine biosynthesis in these cells also retarded S-phase progression (Fredlund, 1997). These results clearly show that polyamines have

critical roles in cell growth and the cell cycle. Polyamines are believed to interact with cellular proteins that regulate the cell cycle (Thomas, 2001).

Polyamine Metabolism

The polyamine metabolic network in both eukaryotes and prokaryotes is tightly regulated, to maintain the balance of intracellular polyamine pools. Cells and bacteria regulate their intracellular polyamine levels by mechanisms that control biosynthesis, degradation, uptake, and excretion.

Polyamine Biosynthesis

Most cells have the ability to synthesize PUT and SPD. Some prokaryotes (such as *Pseudomonas aeruginosa*) lack the ability to synthesize SPM. Some studies also reported a low steady-state concentration of SPM in *P. aeruginosa* (Weaver and Herbst, 1958). The polyamine content in both prokaryotes and eukaryotes is elaborately regulated by biosynthesis, degradation, uptake, and excretion. Interconversions among the fundamental polyamines PUT, SPD and SPM occur to maintain the balance of these polyamines. Biosynthesis and uptake of polyamines are stimulated during cellular responses to growth or proliferative stimuli. On the other hand, when polyamine contents are elevated, degradation and excretion are induced, as is homeostatic inhibition of uptake and biosynthesis.

The fundamental polyamine pathway in most eukaryotes is a complex process (Fig. 1-2). It first involves the conversion of arginine by arginase to ornithine (Luk and Casero, 1987). Ornithine is then decarboxylated by ornithine decarboxylase (ODC) to form PUT. Spermine is synthesized from PUT via consecutive aminopropylations of its amino groups. The first aminopropylation occurs with the participation of the co-substrate, decarboxylated *S*-adenosylmethionine (dcSAM) and spermidine synthase.

As a result, SPD is formed and participates in the next aminopropylation reaction. The second aminopropylation occurs selectively on the aminobutyl end, again using dcSAM as an aminopropyl source. However, this time a different enzyme (spermine synthase) is involved. To produce dcSAM (the aminopropyl group donor), methionine first reacts with ATP to form *S*-adenosylmethionine, which is then decarboxylated by AdoMetDC (Seiler, 1990).

Biosynthesis of putrescine

Mammalian cells and fungi have only one pathway for PUT synthesis. According to this biosynthetic pathway, PUT is formed from ornithine via an enzyme-catalyzed decarboxylation. Ornithine decarboxylase (ODC) directly removes the carboxylic (-COOH) functional group from L-ornithine to form PUT. *L*-Ornithine is biosynthesized from arginine, and is present in human plasma at a concentration of about 85 μM, some of which is obtained from the diet (Morgan, 1999). Ornithine is also a product of the urea cycle. Some from this source may participate in the biosynthesis of PUT.

In addition to the ODC pathway to PUT in eukaryotes, many microorganisms and higher plants possess a second pathway via agmatine (Tabor and Tabor, 1985). Decarboxylation of arginine by arginine decarboxylase produces agmatine. Agmatine is then hydrolyzed by agmatinase (agmatine amidinohydrolase) to form PUT and urea (Fig. 1-3). An additional biosynthetic route to PUT has also been observed in plants via agmatine (Smith, 1985). Agmatine deiminase converts agmatine to *N*-carbamoylputrescine, which is then hydrolyzed by *N*-carbamoylputrescine amidase to form PUT, ammonia and carbon dioxide (Fig. 1-3).

Biosynthesis of SPD

Spermidine is biosynthesized from PUT via an enzyme-catalyzed addition of an aminopropyl group. As discussed earlier, spermidine synthase, also named putrescine aminopropyltransferase, is the enzyme that catalyzes the transfer of an aminopropyl group from SAM to putrescine. Spermidine synthase has been isolated and characterized from different sources such as mammalian cells, plants, and bacteria. Regardless of sources, spermidine synthase (which consists of two identical subunits) accepts PUT as its best subtrate.

Biosynthesis of SPM

The aminopropylation that occurs specifically on the aminobutyl end of SPD gives rise to SPM. This time a different aminopropyltransferase named spermine synthase is involved in the biosynthesis of spermine. Like spermidine synthase, spermine synthase also contains two subunits of equal size; and the enzyme also uses an aminopropyl moiety from dcSAM. However, spermine synthase accepts SPD as its best substrate. It seems that SPM synthesis is confined to eukaryotic systems because of the lack of spermine synthase in prokaryotes.

Ornithine decarboxylase

Ornithine decarboxylase (ODC) is generally a dimer consisting of two identical subunits. Pyridoxal 5'-phosphate (PLP) is derived from pyridoxine (vitamin B_6). It is a necessary electrophilic component for ODC activity. The PLP is always found covalently bound to a lysine residue in the active site of ODC via a Schiff base (imine) linkage (Silverman, 1999). The *L*-Ornithine first forms a Schiff base with PLP (Fig. 1-4), and undergoes decarboxylation via a mechanism similar to the decarboxylation of β-keto acids (Fig. 1-5).

Mammalian species show more than 90% homology in amino-acid sequences of ODC (Morgan, 1999). The similarity in ODC genetic makeup suggests that the structural and enzymatic properties of ODC among mammalian species are very similar. Little similarity exists in amino acid sequences between prokaryotes (e.g., *Escherichia coli*) and eukaryotes (e.g., mammalian cells).

_S_-Adenosylmethionine decarboxylase

S-Adenosylmethionine decarboxylase (AdoMetDC) plays an important role in spermidine and spermine biosyntheses. Decarboxylation of *S*-adenosylmethionine (SAM) by AdoMetDC produces decarboxylated SAM (dcSAM), which serves as an aminopropyl donor for spermidine and spermine biosyntheses.

AdoMetDC contains two unequal subunits (α and β), both of which are necessary for the catalytic activity. Instead of PLP, all known forms of AdoMetDC use a pyruvoyl group which binds covalently to the enzyme via an amide linkage (Poelje, 1990). The decarboxylation activity of this enzyme is similar to that of ODC. SAM first connects with the pyruvoyl group via an imine linkage to form a Schiff base (Fig. 1-6). The Schiff base intermediate then undergoes decarboxylation. In the last step, the dcSAM-pyruvoyl-enzyme complex is hydrolyzed to release dcSAM and the enzyme.

Aminopropyltransferases

Aminopropyltransferase catalyzes the biosynthesis of SPD and SPM by transferring an aminopropyl group from dcSAM to PUT and SPD, respectively (Morgan, 1999). Aminopropyltransferases that catalyze SPD and SPM biosyntheses differ significantly. In mammalian cells, spermidine biosynthesis is catalyzed by spermidine synthase; a putrescine aminopropyltransferase that is highly specific for PUT, and does not use SPD

as an aminopropyl acceptor (Pegg et al., 1981). Spermine biosynthesis is catalyzed by an entirely distinct enzyme spermine synthase; a spermidine aminopropyltransferase that only utilizes SPD as an aminopropyl acceptor (Pajula et al., 1979). Bacterial spermidine synthase also uses SPD to produce SPM *in vitro,* although the aminopropylation reaction is much slower. This explains why some bacteria (such as *E. coli*) do not produce SPM endogenously; and why some pseudomonads produce trace amounts of SPM.

Polyamine Catabolism

Polyamine degradation is an important process in the polyamine metabolic network. Elevation of certain polyamines within a cell can be toxic (Thomas and Thomas, 2001). Thus, any excess polyamine (such as SPD, SPM or other exogenous polyamines imported by the organism) must be degraded to maintain the balance of intracellular polyamine pools. The retrograde pathway progresses from SPM to PUT. Spermine is first N^1-acetylated at the aminopropyl fragment by spermidine/spermine N^1-acetyltransferase (SSAT) using acetyl-CoA as an acetyl source (Casero and Pegg, 1993). The resulting N^1-acetylspermine is then oxidized to imine by polyamine oxidase (PAO). This imine is then hydrolyzed, resulting in 3-acetamidopropanal and SPD (Fig. 1-7) (Binda, 2001). The PAO is a flavin adenine dinucleotide (FAD)-containing enzyme that catalyzes the oxidation of the secondary amino groups of SPM, SPD, and their acetylated derivatives (Binda et al., 2002). In eukaryotes, PAO preferentially oxidizes N^1-acetylspermidine and N^1-acetylspermine into imines that are then hydrolyzed to PUT and SPD, respectively (Morgan, 1999). Spermidine is acetylated at the N-1 position of the aminopropyl end, again by SSAT. Oxidation of N^1-acetylspermidine by PAO, followed by hydrolysis of the resulting imine, results in PUT.

Spermidine/spermine N^1-acetyltransferase

Spermidine/spermine N^1-acetyltransferase (SSAT) initiates the polyamine degradation process. This enzyme catalyzes the transfer of an acetyl group from acetyl-CoA to the aminopropyl moiety of spermine or spermidine. SSAT is known to specifically acetylate the aminopropyl end of spermidine, forming only N^1-acetylspermidine. The rate of PAO activity is usually fast; thus, SSAT-catalyzed acetylation is a rate-determining step in polyamine catabolism. SSAT has been purified from rat, hamster, chicken and human tissues (Casero and Pegg, 1993). Regardless of species, purified SSAT is unstable and particularly sensitive to heat.

Polyamine oxidase

Copper-containing amine oxidases. The bovine plasma diamine oxidase is an example of a copper-dependent amine oxidase. Copper-containing amine oxidases act on spermidine or spermine to produce an aminomonoaldehyde [*N*-(4-aminobutyl)-3-aminopropanal] or a dialdehyde (4,9-diaza-dodecanedialdehyde), respectively, ammonia and hydrogen peroxide (Morgan, 1999). These enzymes directly oxidize polyamines containing primary amino groups, the aminopropyl moiety being more favored.

Flavin adenine dinucleotide-containing amine oxidases. Peroxisomal flavin-dependent PAO is known to oxidize N^1-acetylspermine and N^1-acetylspermidine to imine forms. The PAO is present in most organisms, at levels that significantly exceed those of SSAT (Pegg, 1986). Therefore, any acetylated polyamines formed will be quickly further processed by PAO, making acetylated polyamines usually undetectable. FAD, an important coenzyme of PAO, is derived from riboflavin via an intermediate flavin mononucleotide (FMN) (Fig. 1-8). FAD is a good electron sink (Fig. 1-9) which is reduced with concomitant oxidation of N^1-acetylspermidine and N^1-acetylspermine

(Silverman, 2000).

Importance of Understanding Polyamine Metabolism

Polyamine metabolism has been shown to be a potential target for antiproliferative therapy (Marton and Pegg, 1995). Extensive research efforts have focused on the design and synthesis of inhibitors of ODC, AdoMetDC, and PAO. Ornithine decarboxylase and AdoMetDC are two of the most important enzymes in polyamine assembly; and PAO is a key enzyme in polyamine catabolism. Despite little success in clinical applications with difluoromethylornithine (DFMO), an ODC inhibitor; methylglyoxylbis(guanylhydrazone) (MGBG), an AdoMetDC inhibitor; and *N*,*N'*-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), a PAO inhibitor (Pegg, 1988), these inhibitors (Fig. 1-10) have been used successfully for studying polyamine metabolism. DFMO blocks ODC activity in a dose- and time-dependent manner; completely depletes PUT and SPD; and significantly depletes SPM, in some rabbit-corneal cell types (Du, 2003), indicating that the biosyntheses of PUT by ODC and of SPD from PUT are major routes. MGBG, a potent inhibitor of human AdoMetDC, has no significant impact on AdoMetDC of the human pathogen *Trypanosoma cruzi*, suggesting that this pathogen contains a genetically unique AdoMetDC as an enzyme in the polyamine biosynthetic network (Persson et al., 1998).

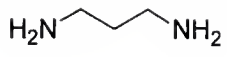
Understanding polyamine metabolism could help explaining abnormal concentrations of polyamines in some pathological conditions. For example, rats induced with central nervous system (CNS) injury showed an abnormal increase in PUT level, and a decrease in SPM and SPD levels, at the injured site. The high PUT level was due to the elevated activity of PAO. The increased PUT was reduced significantly, and

N^1-acetylspermidine increased after treating with MDL 72527, a PAO inhibitor. These observations confirmed that the abnormal increase in PUT after CNS injury was mediated by the SSAT/PAO pathway (Rao, 2000).

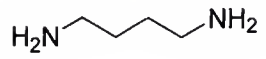
Polyamine Metabolic Pathway Serves as a Therapeutic Target

Polyamines are homeostatically regulated by multiple metabolic pathways such as biosynthesis, uptake, degradation, and excretion. Polyamines have an important role in the proliferation of normal and malignant cells (Davidson, 1999). Elevation of polyamine concentration is not restricted to malignant conditions, because this phenomenon has also been found in body fluids of patients with CF, and during pregnancy (Russel, 1978; Wallace, 2003). However, many studies show a clear link between increased polyamine content and cancer-cell activities. High concentrations of polyamines were also found in tissues with bacterial (Maita, 1990) and parasitic infections (Bacchi, 1999).

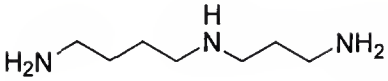
The abnormal increase in polyamine biosynthesis in neoplastic tissues has made the polyamine metabolic network a potential target in anticancer (Marton, 1995) and antibacterial strategies (Wang, 1991).



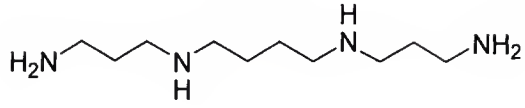
Diaminopropane (DAP)



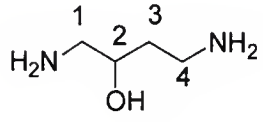
Putrescine (PUT)



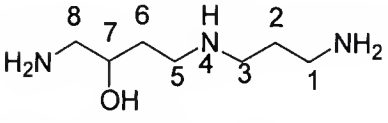
Spermidine (SPD)



Spermine (SPM)



2-Hydroxyputrescine (2-OH-PUT)



7-Hydroxyspermidine (7-OH-SPD)

Figure 1-1. Naturally occurring polyamines. Numbers in the structures of 2-OH-PUT and 7-OH-SPD indicate the positions of carbon molecules.

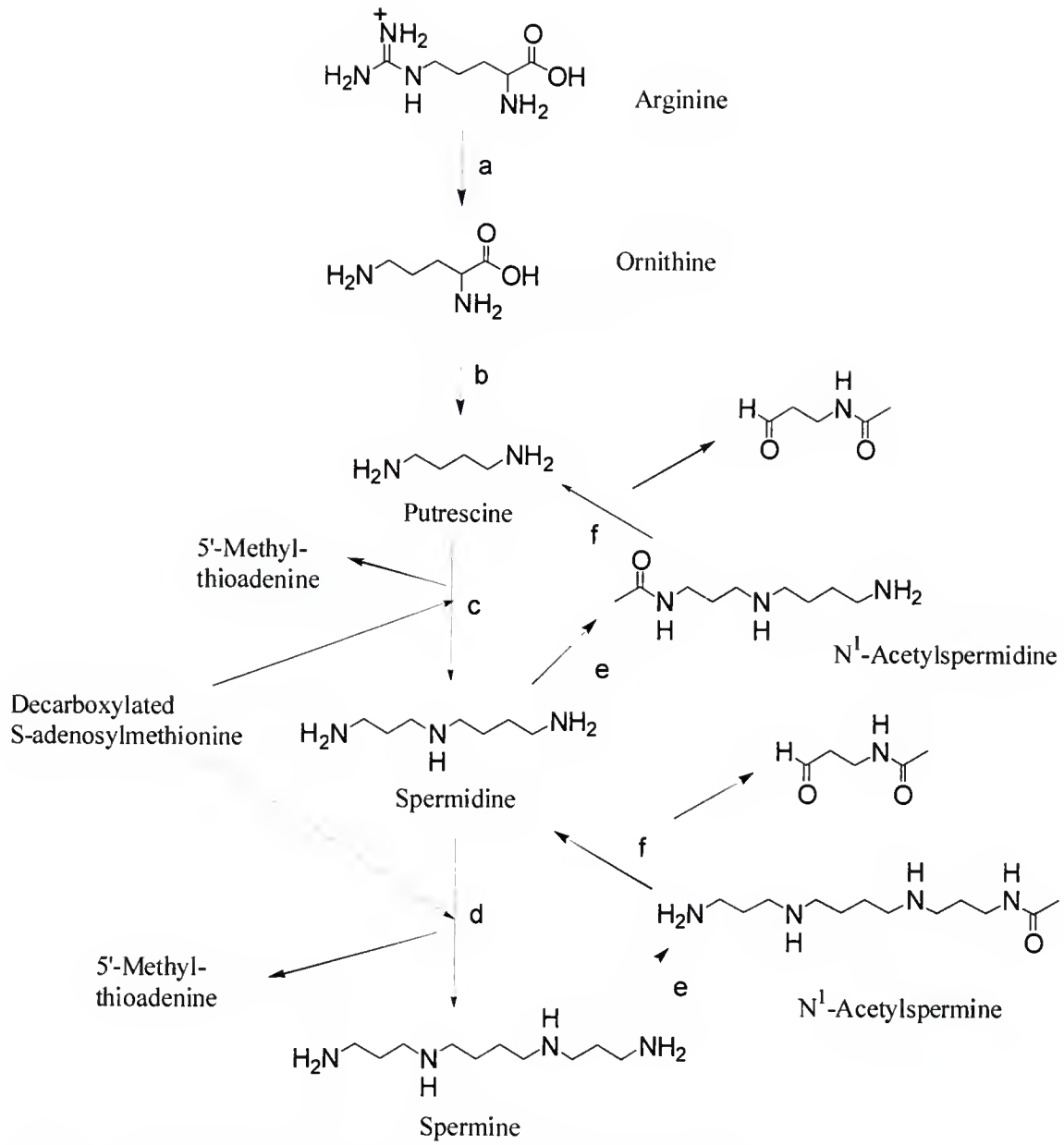


Figure 1-2. Fundamental polyamine metabolism pathway in eukaryotes.
(a) Arginase, (b) Ornithine decarboxylase, (c) Spermidine synthase, (d) Spermine synthase, (e) Spermidine/Spermine N^1-acetyltransferase, (f) Polyamine oxidase, (g) S-adenosylmethionine synthase, (h) S-adenosylmethionine decarboxylase.

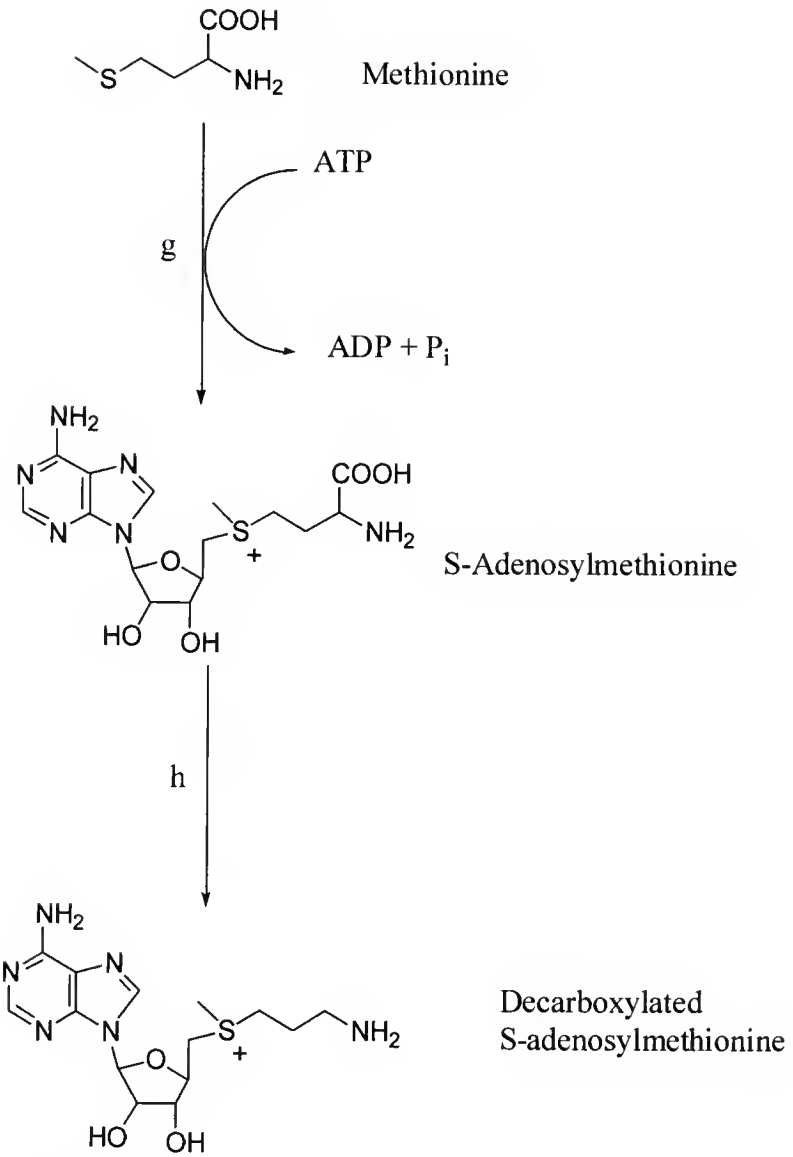


Figure 1-2. Continued

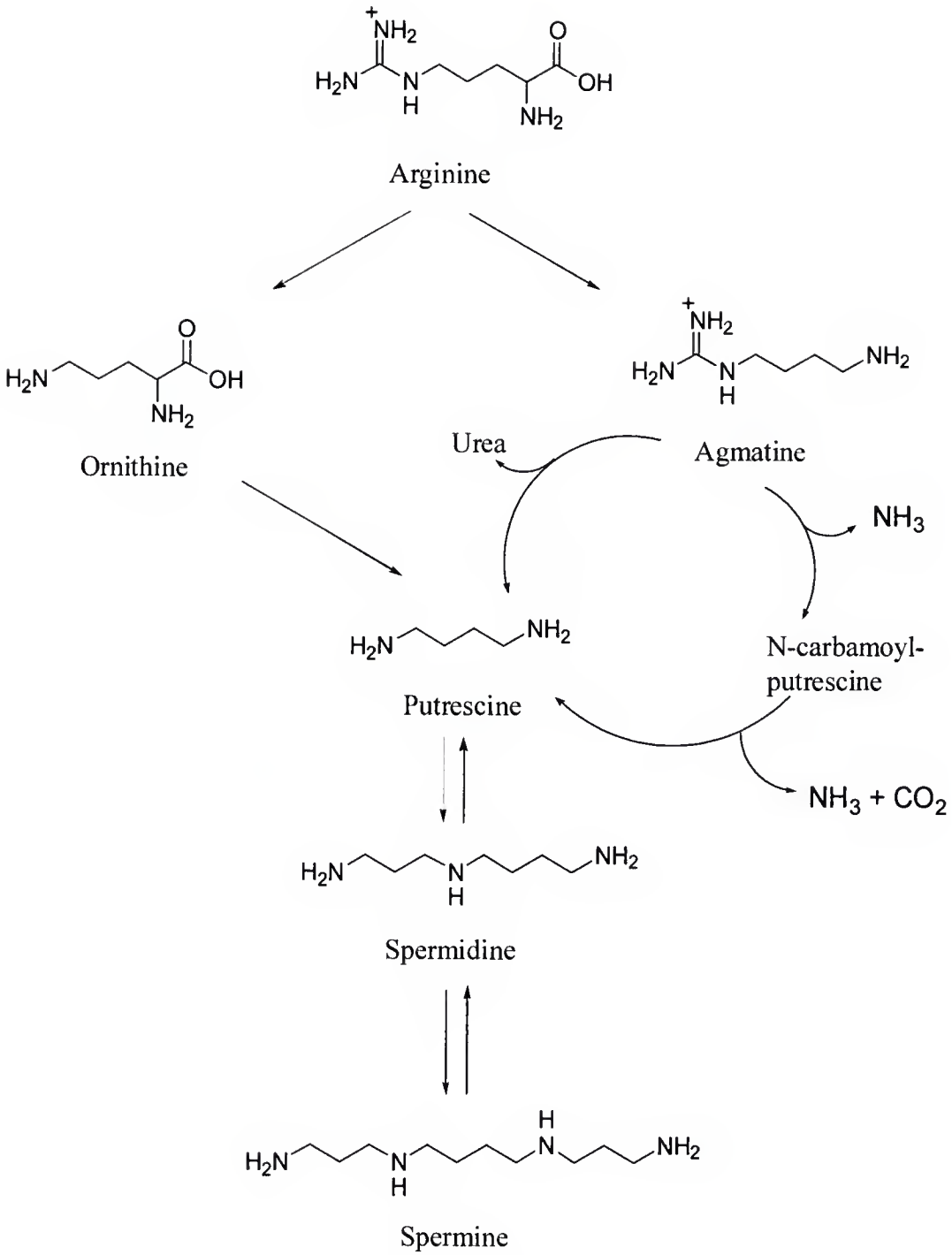


Figure 1-3. Polyamine metabolism in plants and bacteria

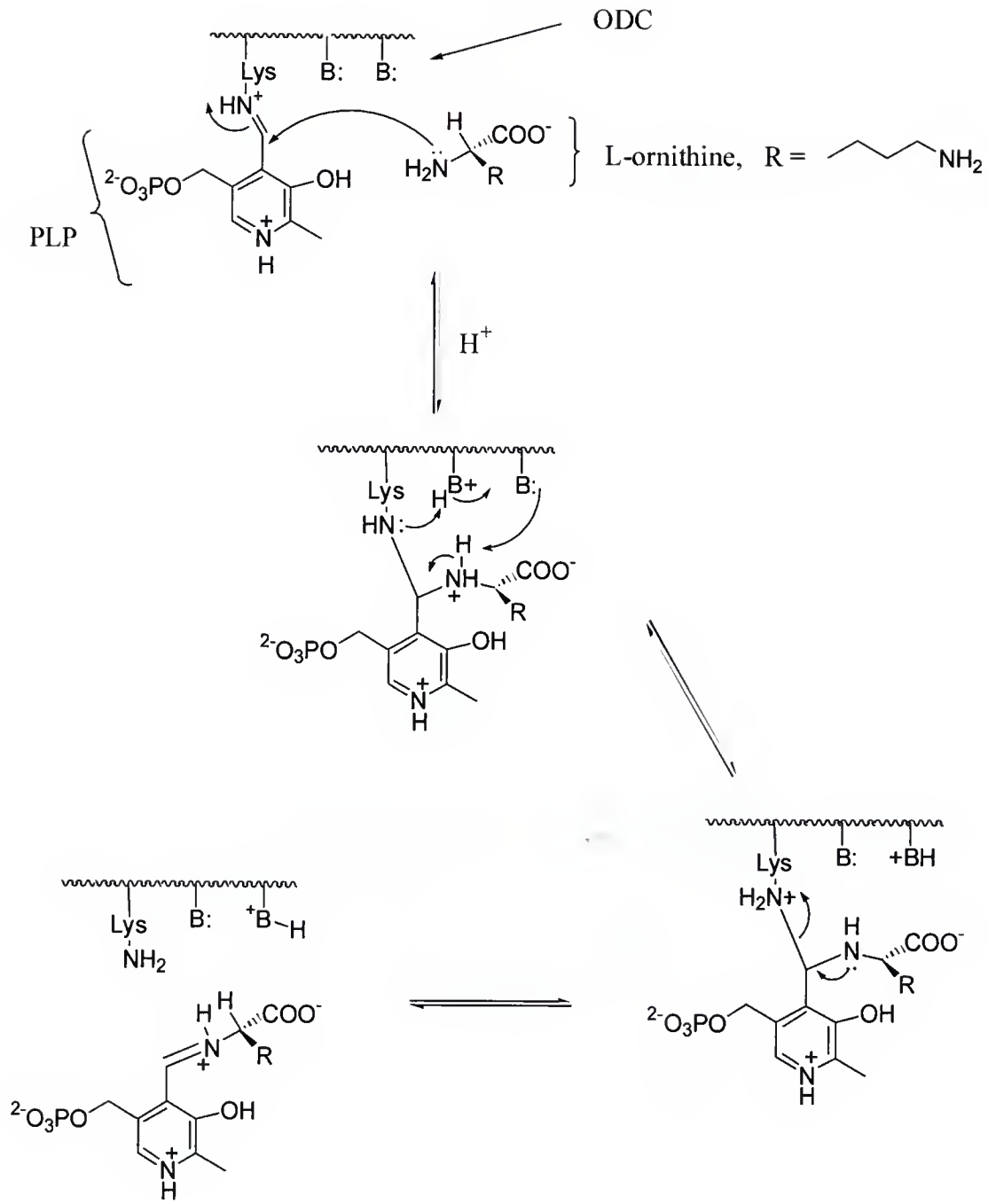


Figure 1-4. Formation of the Schiff base between L-ornithine and PLP (PLP: coenzyme of ODC). B: a base in ODC active site

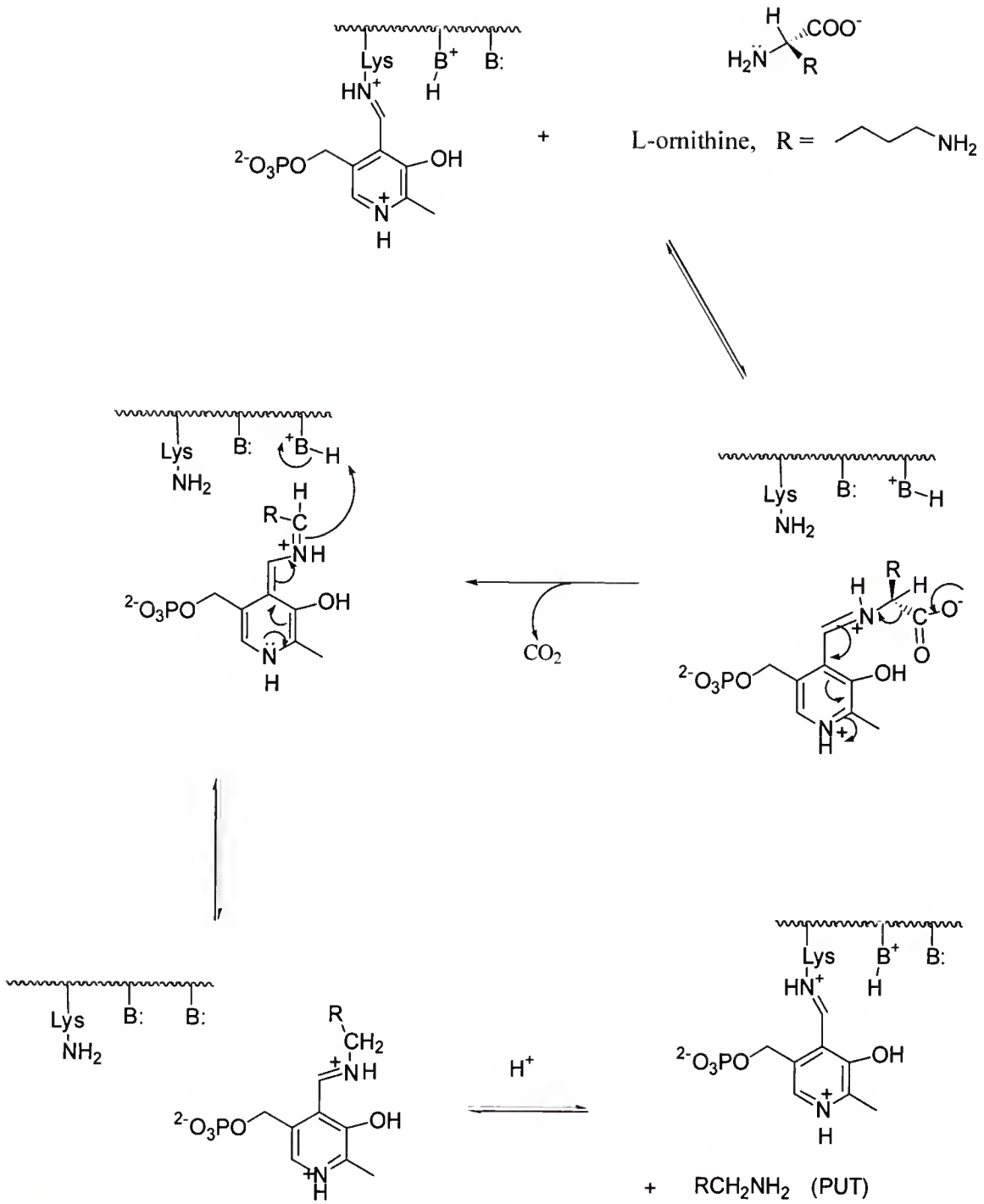


Figure 1-5. Proposed mechanism of ornithine decarboxylation

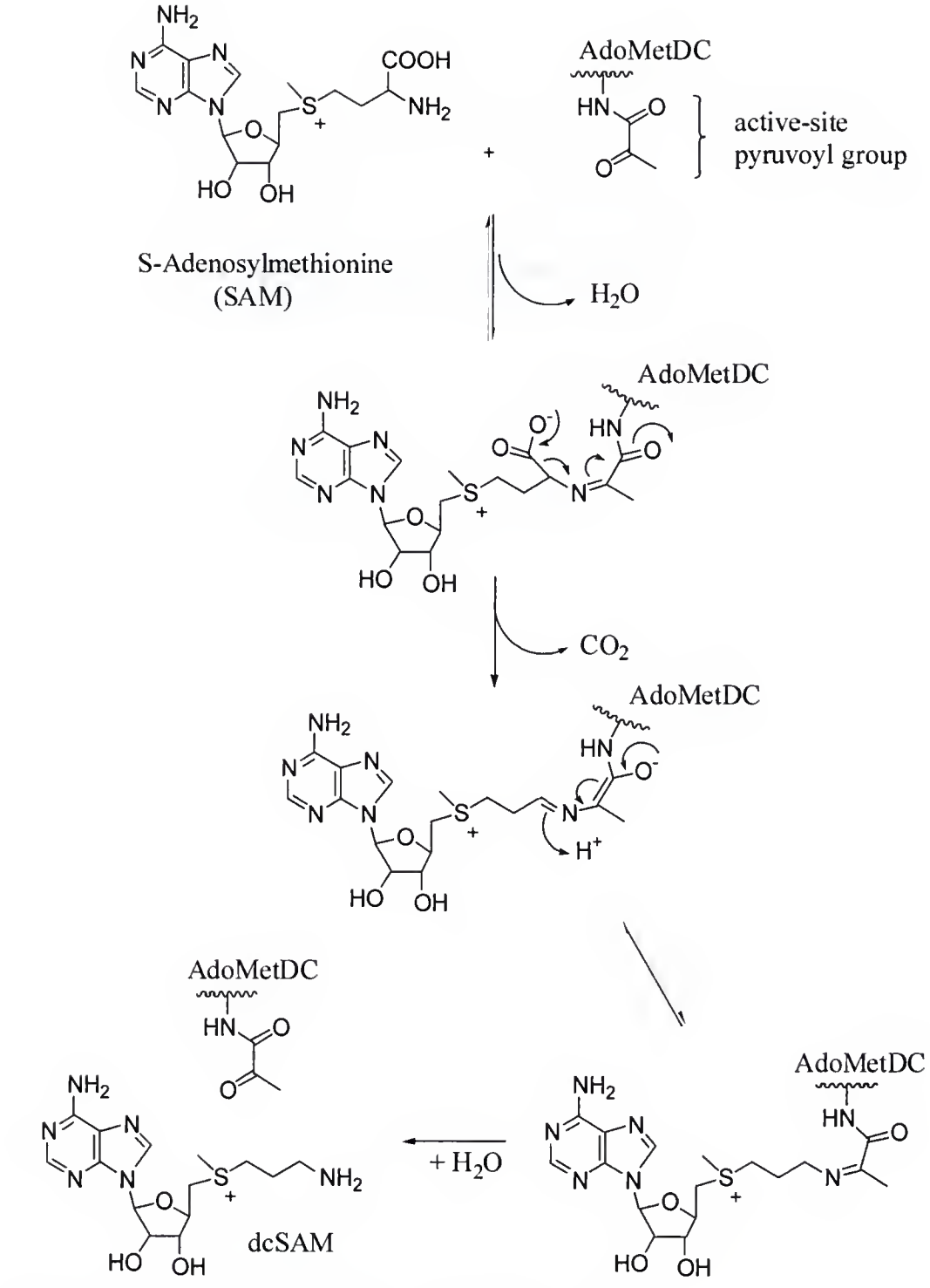


Figure 1-6. Proposed mechanism of AdoMetDC activity

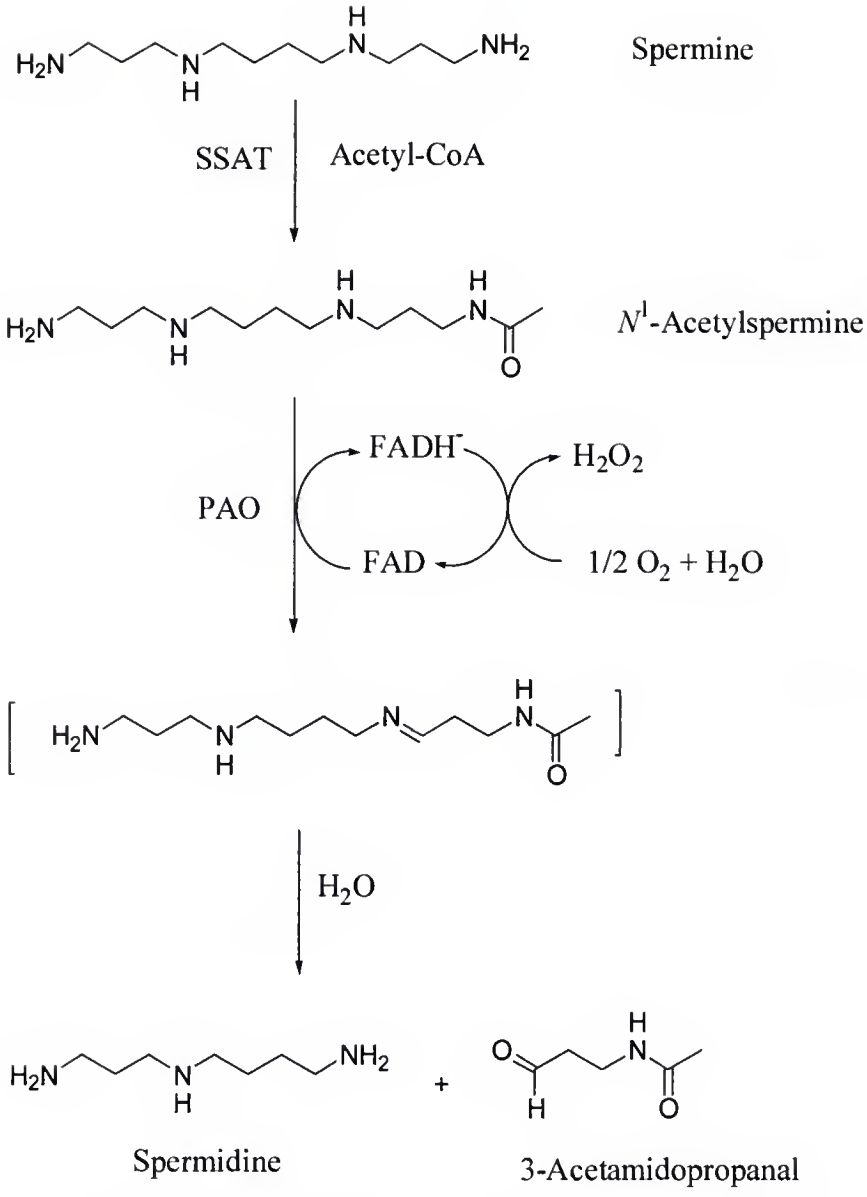
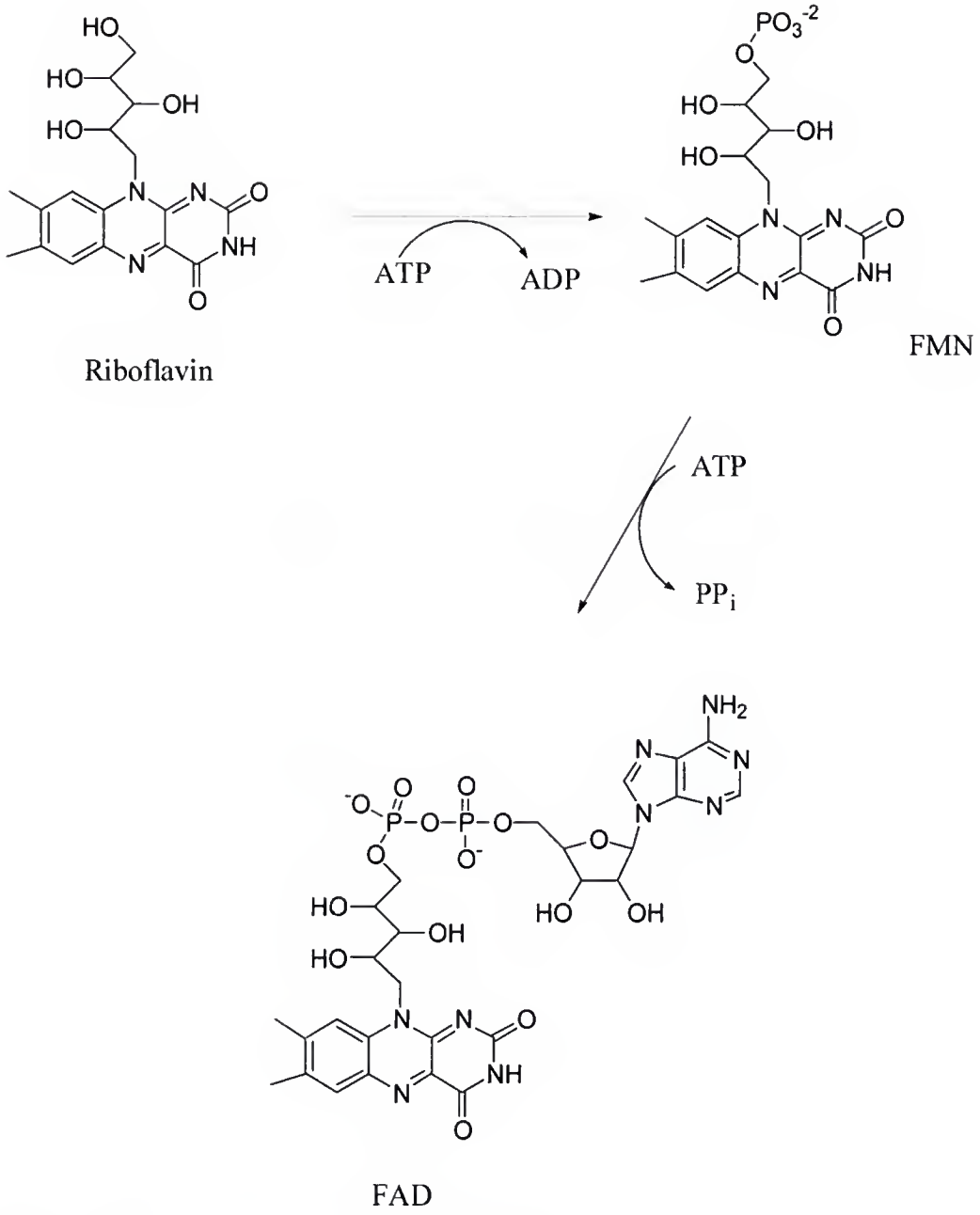


Figure 1-7. Metabolic breakdown from spermine to spermidine by SSAT/PAO. SSAT: spermidine/spermine N^1-acetyltransferase. PAO: polyamine oxidase. FAD: flavin adenine dinucleotide.

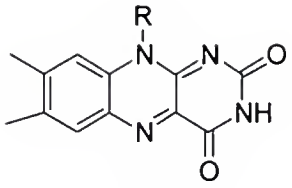


Riboflavin

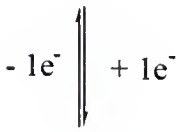
FMN

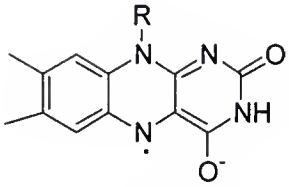
FAD

Figure 1-8. Biosynthesis of FAD from riboflavin

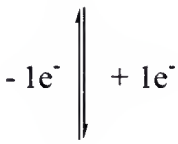


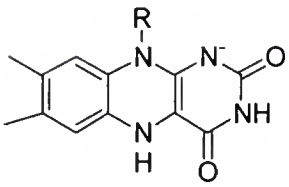
FAD





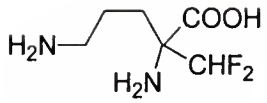
$[FAD^{\cdot}]^{-}$



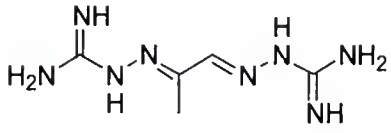


$[FADH]^{-}$

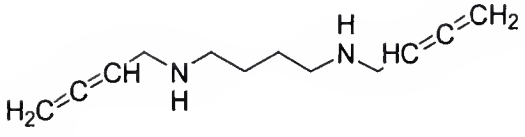
Figure 1-9. Different oxidation states of FAD



DFMO, an irreversible inhibitor of ODC



MGBG, an AdoMetDC inhibitor



MDL 72527, a PAO inhibitor

Figure 1-10. Structures of some polyamine metabolic enzyme inhibitors

CHAPTER 2
POLYAMINE CONTENTS AND POLYAMINE METABOLISM IN PSEUDOMONADS: LITERATURE REVIEW

Polyamine metabolism in some prokaryotes is somewhat different from that in animal cells. In addition to the metabolic pathway described above, some microorganisms (e.g., *P. aeruginosa,* also possess a second pathway via agmatine) (Fig. 2-1). In *P. aeruginosa*, PUT can be biosynthesized either directly from ornithine by ODC or indirectly from arginine via arginine decarboxylase (ADC). Arginine is decarboxylated by ADC to form agmatine. Agmatine is then hydrolyzed by agmatinase to form PUT, with the elimination of urea (Morgan, 1999). The genes of two enzymes ODC and ADC of wild type *P. aeruginosa* strain PAO1 were identified as *speC* and *speA* (Nakada and Itoh, 2003). It was also reported that the activities of these two decarboxylases were similar, and each individual decarboxylase appeared to direct sufficient polyamine biosynthesis for normal growth.

In rapidly growing organisms including cancer cells and bacteria, the demand for polyamines is high. The polyamine pathway has been elucidated for a relatively small number of organisms, and there is significant interspecies variation (Casero and Woster, 2001). For example, in *P. acidovorans,* 2-OH-PUT, PUT, SPD, and 7-OH-SPD were identified but in *P. aeruginosa*, only PUT, SPD, and trace amounts of DAP and SPM were found (Table 2-1) (Bitonti et al., 1982; Kallio et al., 1981; Weaver and Herbst, 1958). Additional polyamines present in selected pseudomonads are also listed in Table 2-1.

Pseudomonads are allocated to three different subclasses of *proteobacteria*, α, β, and γ, by their differences in polyamine distribution patterns. PUT and homospermidine (HSPD) make up the α subclass. PUT, 2-OH-PUT, and SPD are in the β subclass and PUT, DAP and SPD belong to the γ subclass (Busse and Auling, 1988). *Burkholderia cepacia*, DSM 50181, is a common pathogenic bacterial strain that produces a large amount of 2-OH-PUT and a small amount of HSPD in addition to PUT and SPD. *P. aeruginosa* belongs to the γ subclass, which contains considerable amount of PUT, SPD, and a small amount of DAP. Spermine is also present in *P. aeruginosa* at a low steady state concentration. To date, there is no evidence of hydroxypolyamines (i.e., 2-OH-PUT or 7-OH-SPD) in bacteria of the γ subclass.

Pseudomonas sp Strain Kim

In *P. sp* strain Kim, a member of the β subclass (Table 2-1), PUT, 2-OH-PUT and SPD were identified (Rosano et al., 1989), but whether 7-OH-SPD was present in these bacteria was not reported. The steady state level of SPD was low because of the efficient conversion of SPD to PUT and 2-OH-PUT that ultimately prevented accumulation of SPD in the bacteria. However, whether 2-OH-PUT was derived from SPD directly or via PUT was unclear. Incubation of ^{14}C-SPD (12.5 μM) with growing log phase bacteria for 6 h resulted in the formation of radioactive PUT and 2-OH-PUT. These results suggested that *P. sp* strain Kim contains N^1-SPD acetyltransferase and PAO, the enzymes needed to convert SPD to PUT. When exposed to MGBG (an AdoMetDC inhibitor) and ^{14}C-SPD (12.5 μM) simultaneously, the amounts of radioactive PUT and 2-OH-PUT found in these same bacteria increased 5-7 fold. Note that in the presence of MGBG, the biosynthesis of endogenous SPD was reduced, causing a decrease in the conversion from

SPD to PUT and 2-OH-PUT. Therefore, these bacteria increased the processing of ^{14}C-SPD to form radiolabeled polyamines PUT and 2-OH-PUT to stabilize the polyamine pools.

In a separate experiment, ^{14}C-SPD was added to growing log phase *Pseudomonas* sp strain Kim with and without MDL 72527, a PAO inhibitor, added to the bacteria nutrient broth. MDL 72527 only reduced the conversion of radioactive SPD to radioactive PUT and radioactive 2-OH-PUT by 40%, and it had no significant impact on total intracellular levels of PUT and 2-OH-PUT. Rosano et al. suggested that 40% of radiolabeled SPD must have been quickly converted to radioactive PUT and 2-OH-PUT before the inhibition of PAO by MDL 72527 took effect. It was interesting to note that inhibitor MDL 72527 did not significantly affect the levels of non-radioactive PUT and 2-OH-PUT. The authors suggested that PUT and 2-OH-PUT are important in the bacterial growth, and these polyamines could have been synthesized under homeostatic control (Rosano et al., 1989).

Pseudomonas acidovorans

In addition to 2-OH-PUT, PUT and SPD, 7-OH-SPD (1-3 μg/wet wt cells) was identified in *P. acidovorans,* a member of the β subclass (Rosano et al., 1978) (Table 2-1). It was demonstrated that 7-OH-SPD could be synthesized enzymatically from 2-OH-PUT by cell-free preparations from *E. coli* or *P. acidovorans*, which contain an aminopropyltransferase called SPD synthase. The presence of 7-OH-SPD, but not 6-OH-SPD, as a naturally occurring polyamine suggested that SPD synthase reacts preferentially with the amine distal to the hydroxyl group (Fig. 2-2). The SPD synthase from *P. acidovorans* was found to be capable of synthesizing both SPD and 7-OH-SPD

from their respective substrates. Recall that PUT serves as a substrate for SPD synthase and 2-OH-PUT is a derivative of PUT with a hydroxyl substituent at the C-2 position. This finding is consistent with the observation that in addition to PUT, spermidine synthase can utilize PUT derivatives with a variety of substituents at the C-2 position including hydroxyl and methyl groups (Sarhan et al., 1987).

Pseudomonas thermocarboxydovorans

Pseudomonas thermocarboxydovorans is also a member of the β subclass. However, the polyamine content of these bacteria differs slightly from *P. acidovorans*. It also contains DAP (Hamana and Matsuzaki, 1990). It was suggested that, like *P. acidovorans*, 7-OH-SPD is synthesized from 2-OH-PUT as a result of preferential action of the aminopropyltransferase with the amine distal to the hydroxyl group (Fig. 2-2).

Aminopropyltransferase catalyzes the biosynthesis of SPD and SPM by transferring an aminopropyl group from dcSAM to PUT and SPD, respectively (Morgan, 1999). There appear to be significant differences in the aminopropyltransferases that catalyze SPD and SPM biosyntheses. In mammalian cells, SPD biosynthesis is catalyzed by spermidine synthase, a putrescine aminopropyltransferase, which is highly specific for PUT and does not use SPD as an aminopropyl acceptor (Pegg et al., 1981). Spermine biosynthesis is catalyzed by an entirely distinct enzyme spermine synthase, a spermidine aminopropyltransferase, which only utilizes SPD as an aminopropyl acceptor (Pajula et al., 1979). Spermine can be biosynthesized in some bacteria despite the absence of spermine synthase since bacterial spermidine synthase can also utilize spermidine as a substrate. However, the spermine biosynthetic reaction catalyzed by spermidine synthase

is much slower than by spermine synthase. Some bacteria, such as *E. coli,* do not produce SPM endogenously, yet some pseudomonads produce trace amounts of SPM.

Burkholderia cepacia

In a clinical setting, *B. cepacia* (formerly classified as *P. cepacia*) often infects patients with cystic fibrosis after a lengthy colonization by *P. aeruginosa*. Patients with severe respiratory impairment, who are considered for lung transplantation, are also at a high risk for infection. Acquisition of *B. cepacia* after lung transplantation was markedly associated with morbidity and mortality (Steinbach et al., 1994). *B. cepacia* is also a member of the β subclass (Table 2-1) and contains large amounts of OH-PUT (24.7-30.5 μmol/g dry weight) and PUT (43.3-58.2 μmol/g dry weight) (Busse and Auling, 1988). The pathogen also contains cadaverine (8.2-14.6 μmol/g dry weight) and a small amount of SPD (0.3 μmol/g dry weight). Whether 7-OH-SPD was measured or not was unclear from these available studies. In the presence of abundant endogenous 2-OH-PUT in *B. cepacia*, 7-OH-SPD was expected to be biosynthesized by spermidine synthase. Perhaps the efficient conversion from 7-OH-SPD to 2-OH-PUT resulted in an undetectable steady-state level of 7-OH-SPD.

Pseudomonas aeruginosa

Biology

P. aeruginosa is one of the best known species among pseudomonads. It grows well in all of the common culture media at a temperature range of 30-37 ^{0}C. Although aerobic conditions are required, some strains of *P. aeruginosa* can grow in anaerobic conditions with the presence of nitrate. In these cases, the oxygen in nitrate serves as the

electron acceptor. In common with other pseudomonads, *P. aeruginosa* can utilize a large number of organic substrates as carbon sources.

P. aeruginosa is usually a straight bacillus. The bacterium is motile by a single polar flagellum. The flagellum is also important in bacterial virulence and biofilm (bacterial protective matrix) formation. Bacterial biofilm is a large, flexible, but sturdy matrix. It contains water channels for the intake of nutrients and export of wastes. Each bacterium is between 1.5 to 4.0 μm long and about 0.5 μm in diameter. *Pseudomonas. aeruginosa* produces fluorescein (a green fluorescent pigment) and pyocyanin (a phenazine blue pigment). Fluorescein is found in several pseudomonads while pyocyanin is uniquely found in *P. aeruginosa.*

P. aeruginosa is first formed in the biofilm layer that protects the bacteria from extreme physical, chemical, and biological conditions. Upon maturing, the planktonic bacteria are released from the biofilm and if survived, they will proliferated quickly (doubling time = 40 min), cause acute infections, and develop new biofilms. Bacteria in biofilm are 50-5000 times more resistant to anti-pseudomonal drugs compared to the planktonic bacteria.

Pathogenicity

Pseudomonas aeruginosa was chosen for this study because infections caused by this bacterium are of major concern in medical facilities, particularly in intensive care and burn units. *Pseudomonas aeruginosa* is a Gram-negative bacterium which possesses a complex and sturdy cellular membrane structure (Wilson and Dowling, 1998). Sites of infection include the CNS, bones, joints, skin, eyes, ears, gastrointestinal tract, urinary tract and lungs. *Pseudomonas aeruginosa* is an opportunistic organism, but is relatively

harmless to healthy humans. However, immunocompromised patients who have had surgery, transplant, cancer, HIV, and cystic fibrosis are at high risk for infection (Fick, 1993). *Pseudomonas aeruginosa* is capable of utilizing a wide variety of carbon sources, thus contributing to its virulence (Delden and Iglewski, 1998). Once infection is established, *P. aeruginosa* produces toxic proteins that cause extensive tissue damage and also interfere with human defense mechanisms. These proteins range from potent toxins that enter and kill host cells to degradative enzymes that permanently disrupt the cell membranes and connective tissues in various organs (Todar, 2002). In patients with cystic fibrosis (CF), *P. aeruginosa* successfully colonize the respiratory tract since it is able to produce a highly protective capsule made of the mucoid polysaccharide alginate. This allows the bacteria to adhere better to the lining of the lungs and to resist phagocytosis by immune cells. Antibiotics do not effectively eradicate *P. aeruginosa* from the lungs in these patients (Wilson and Tsang, 1994).

Current treatment of *P. aeruginosa* includes the usage of many classes of antibiotics including cephalosporins, aminoglycosides and carbapenems (Jones and Varnam, 2002; Korvick and Yu, 1991). Development of resistance to antibiotics is a major problem in fighting bacterial infection. Some pseudomonad strains can inactivate the drugs that threaten them by using certain enzymes to modify the drug (Poole, 2002), while other strains can use their active efflux systems to export various antibiotics (Levy, 2002).

Polyamine Contents and Metabolism

Pseudomonas aeruginosa is a member of the γ subclass (Table 2-1). It contains DAP (2.9 μmol/g dry weight), PUT (34 μmol/g dry weight), and SPD (8.6 μmol/g dry

weight). A study also reported that *P. aeruginosa* contains trace amount of spermine (Weaver, 1958).

Monofluoromethylornithine (MFMO) at 2 mM can inhibit the activity of ODC in *P. aeruginosa* by 98%. Difluoromethylarginine (DFMA) at 1 mM can inhibit arginine decarboxylase (ADC) activity by 94% but cannot inhibit ODC activity (Kallio et al., 1981). Although MFMO and DFMA were efficiently transported into bacteria from the nutrient broth, these drugs individually could not inhibit PUT biosynthesis. In order to inhibit PUT biosynthesis, it is necessary to use both drugs to block both ODC and ADC activities. However, because of the rapid turnover of ODC and ADC, a high concentration of drugs is needed to achieve significant inhibition on these enzymes.

Addition of a combination of three drugs, MFMO (2 mM), DFMA (2.5 mM) and DCHA (dicyclohexyl ammonium sulfate, a competitive inhibitor of SPD synthase) (5 mM), to cultures of *P. aeruginosa* slowed the bacterial growth (Bitonti et al., 1984). The doubling time increased from 42 min to 62 min. Intracellular concentrations of PUT and SPD decreased significantly to 41% and 19% of control levels, respectively. The normal growth rate was restored by the addition of 0.1 mM SPD or 5 mM PUT. Addition of SPD resulted in the increase of intracellular PUT, and addition of SPM resulted in increases of both PUT and SPD. On the other hand, addition of PUT only increased the intracellular PUT but had no effect on SPD or SPM. It was clear that SPD and SPM were more effective in reversing the growth inhibition. SPD at a low concentration (0.1 mM) was found to be sufficient to reverse the growth inhibition while a large amount of exogenous PUT (5 mM) was required to reverse the growth inhibition (Bitonti et al.,

1984). However, it was still unclear from this study whether or not SPD itself was responsible for the growth recovery.

P. aeruginosa does not produce any 2-OH-PUT or 7-OH-SPD. However, this bacterium can utilize these hydroxypolyamines without affecting its growth. In the next chapter, we will describe how this microorganism incorporated the hydroxypolyamines into its polyamine metabolic network.

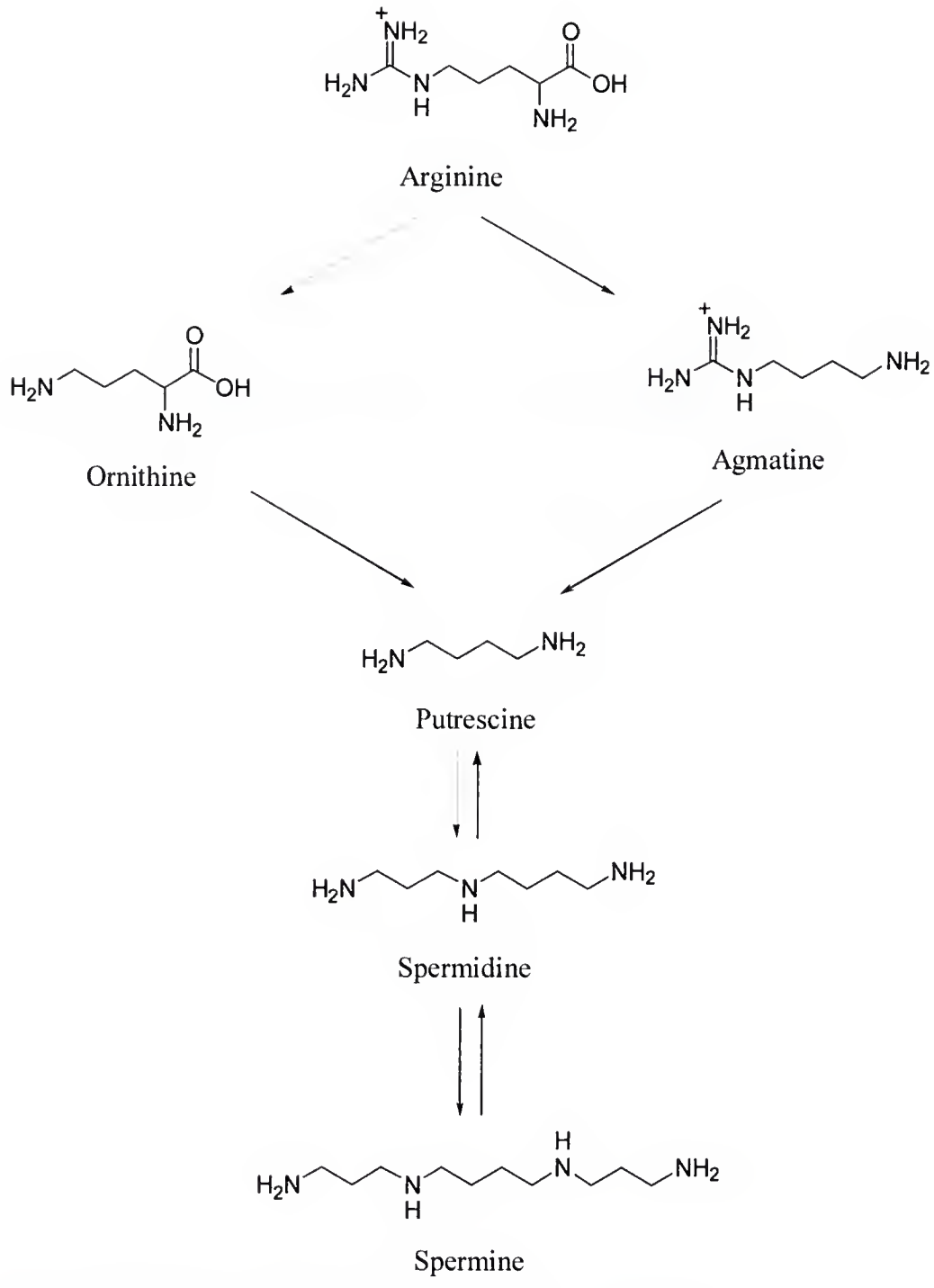


Figure 2-1. Polyamine metabolism in *Pseudomonas aeruginosa*

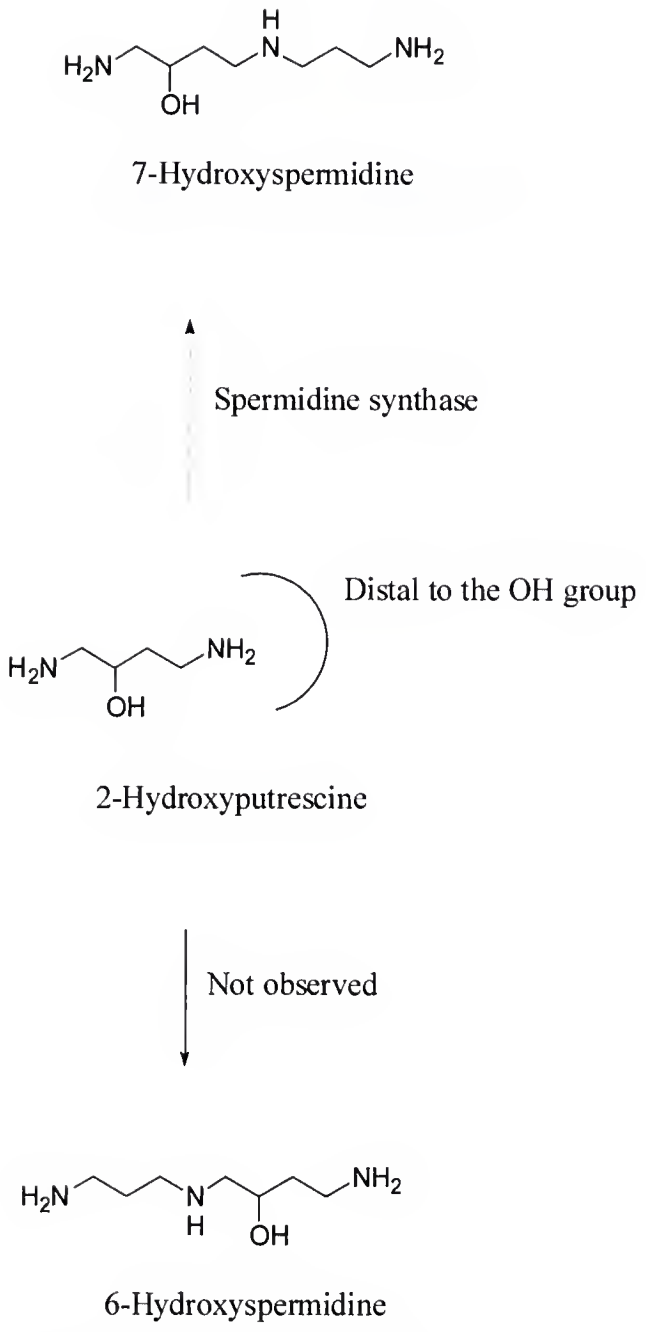


Figure 2-2. Biosynthesis of 7-hydroxyspermidine observed in *P. acidovoran* and *P. thermocarboxydovorans*.

Table 2-1. Polyamine contents of selected pseudomonads

Species	**Subclass**	**Naturally occurring polyamines (μmole/g dry weight unless specified)**
P. aminovorans	α	PUT (12.2) SPD (0.8) HSPD (53.0) SPM (0.8)
P. aeruginosa	γ	DAP (2.9) PUT (34.0) SPD (8.6)
P. acidovorans	β	2-OH-PUT (57.1) PUT (57.8) SPD (0.6) 7-OH-SPD (1-3 μg/g wet wt)
Burkholderia cepacia	β	Cadaverine (8.2-14.6) 2-OH-PUT (24.7-30.5) PUT (43.3-58.2) SPD (0.3)
P. sp strain Kim	β	PUT (13.3 nmol/OD unit) 2-OH-PUT (9.08 nmol/OD unit) SPD (trace amount)
P. thermocarboxydovorans	β	DAP (0.100 μmol/g wet wt) PUT (0.500 μmol/g wet wt) SPD (0.760 μmol/g wet wt) 2-OH-PUT (0.129 μmol/g wet wt) 7-OH-SPD (0.400 μmol/g wet wt)

CHAPTER 3
PROCESSING OF 2-HYDROXYPUTRESCINE AND 7-HYDROXYSPERMIDINE IN *PSEUDOMONAS AERUGINOSA*

In the previous chapter, we discussed hydroxypolyamines in a few pseudomonads. Unlike pseudomonads in the β subclass, *P. aeruginosa,* a member of the γ subclass, does not contain hydroxypolyamines. We would like to understand how *P. aeruginosa* incorporates 2-OH-PUT and 7-OH-SPD into its polyamine biosynthetic network as well as its catabolic machinery.

Hydroxypolyamines

Free forms of 2-OH-PUT and 6-OH-SPD are not observed in living organisms. However, they incorporate in some naturally occurring compounds. The 2-OH-PUT is a component of hypusine (a naturally occurring compound that is present in bovine brain tissue) (Shiba, 1971), while 6-OH-SPD is a vital component of a cytotoxic marine compound. Hypusine is also present in various organs (such as liver, kidney, spleen, heart, and lung) of Wistar rats (Nakajima, 1971). Unlike 2-OH-PUT and 6-OH-SPD, 7-OH-SPD is a naturally occurring hydroxypolyamines present in some pseudomonads as discussed in chapter 2. The three hydroxypolyamines were prepared previously in our laboratory; and thus, the authentic samples were used as standards for analytical analyses.

Impact on Bacterial Growth

As the exposure concentrations of 2-OH-PUT increased from 0.05 to 1.0 mM, the lag time (the time it took before the bacteria started to enter logarithmic phase) gradually increased (Fig. 3-1). Even at high concentration, i.e. 1.0 mM for 2-OH-PUT and 1.0 mM

for 7-OH-SPD, these two hydroxypolyamines did not have any significant impact on bacterial growth by stationary phase. When grown without these exogenous polyamines, the bacteria usually started the logarithmic phase at t = 11 h, and reached the stationary phase by 18 h. In the presence of each hydroxypolyamine at 1.0 mM, the bacteria started the logarithmic phase at 15 h, and reached the stationary phase by 23 h (Fig. 3-2). In addition, the maximum bacterial turbidity usually decreased to about 80 % of control.

Processing of Hydroxypolyamines and Impact on Bacterial Polyamine Contents

Incubation of *P. aeruginosa* with 2-OH-PUT resulted in decreasing intracellular concentrations of PUT and SPD. The depletion of these bacterial natural polyamines occurred in a dose-dependent manner. At a concentration of 1.0 mM, 2-OH-PUT reduced PUT and SPD levels to 66% and 69% of control, respectively. It was also noticeable that as the exposure concentration of 2-OH-PUT increased, the intracellular level of 2-OH-PUT also increased. In addition, 2-OH-PUT was converted to both 7-OH-SPD and 6-OH-SPD (Fig. 3-3). The formation of each of the two hydroxypolyamines, 7-OH-SPD and 6-OH-SPD, were dependent of the exposure concentration of 2-OH-PUT (Fig. 3-4). The low steady state levels of 7-OH-SPD and 6-OH-SPD (< 1.0 nmol/OD unit) were probably due to the dynamic back conversion to 2-OH-PUT, or perhaps because these hydroxypolyamines are not important for the growth of *P. aeruginosa*. Unlike *P. acidovorans* or *P. thermocarboxydovorans*, it was surprising that 6-OH-SPD was biosynthesized from 2-OH-PUT in *P. aeruginosa*. Spermidine synthase in this bacterium must have catalyzed the aminopropylation from either amino end of 2-OH-PUT. Careful inspection of the HPLC chromatographs further confirmed the formation of 6-OH-SPD and 7-OH-SPD in *P. aeruginosa* grown in the

presence of 2-OH-PUT. In the untreated bacteria, a HPLC chromatograph showed no detectable level of any of the hydroxypolyamines (Fig. 3-5). On the other hand, in the bacteria incubated with 1.0 mM 2-OH-PUT for 16 h (mid log phase), 6-OH-SPD and 7-OH-SPD were formed. According to a HPLC chromatograph (Fig. 3-6), 6-OH-SPD eluted at 20.001 min (peak # 9) with an area under the curve of 203786 (~ 2.1% of PUT area) while 7-OH-SPD eluted at 19.598 min (peak # 8) with an area of 65543 (~ 0.7% of PUT area). These interesting observations have stimulated further studies on polyamine metabolic activity in prokaryotes and eukaryotes with respect to exogenous polyamine analogues.

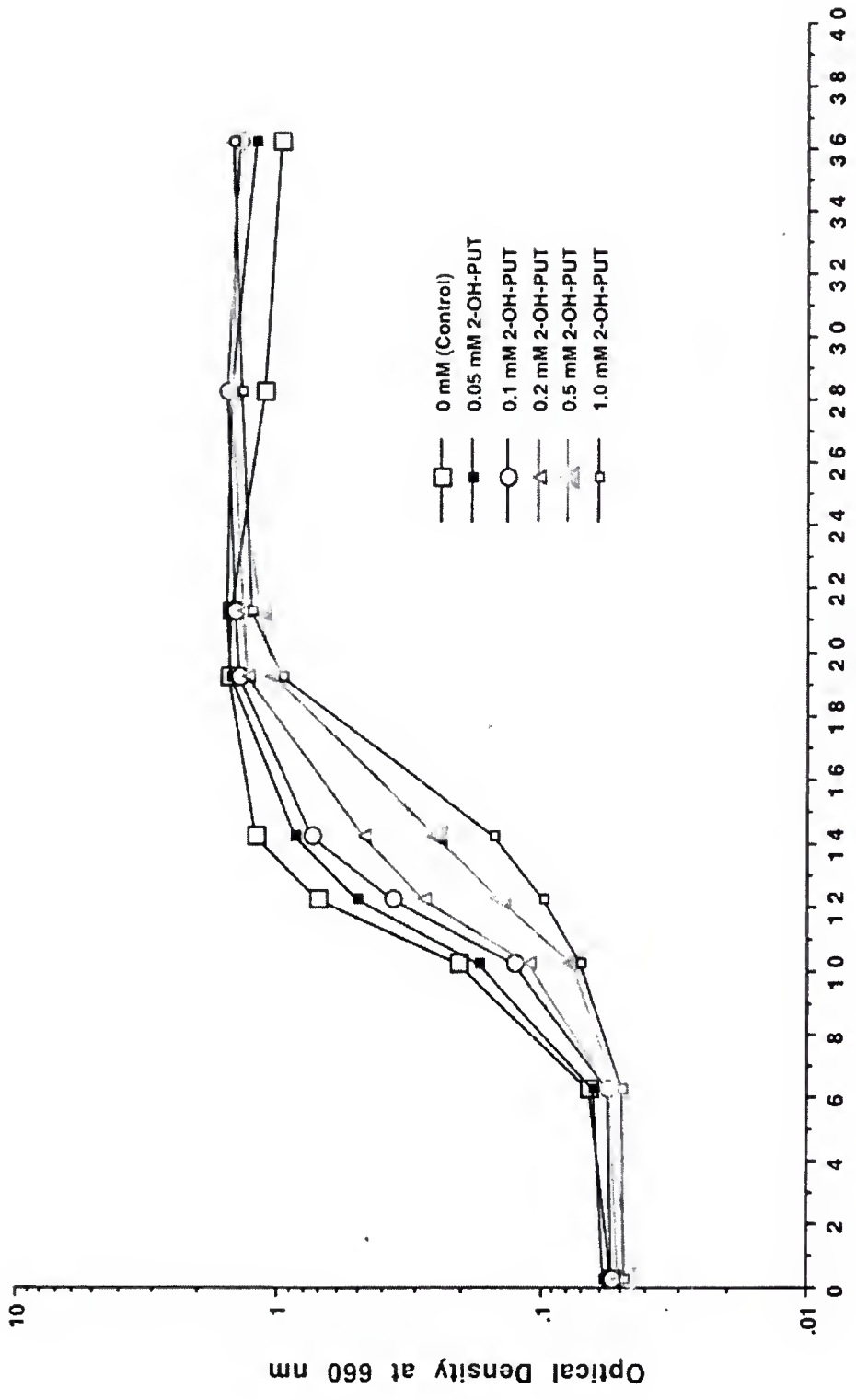


Figure 3-1. Effect of 2-OH-PUT on the growth of *P. aeruginosa*. Each experiment was performed in duplicate and repeated at least once. Errors in OD_{660} readings were within 5%.

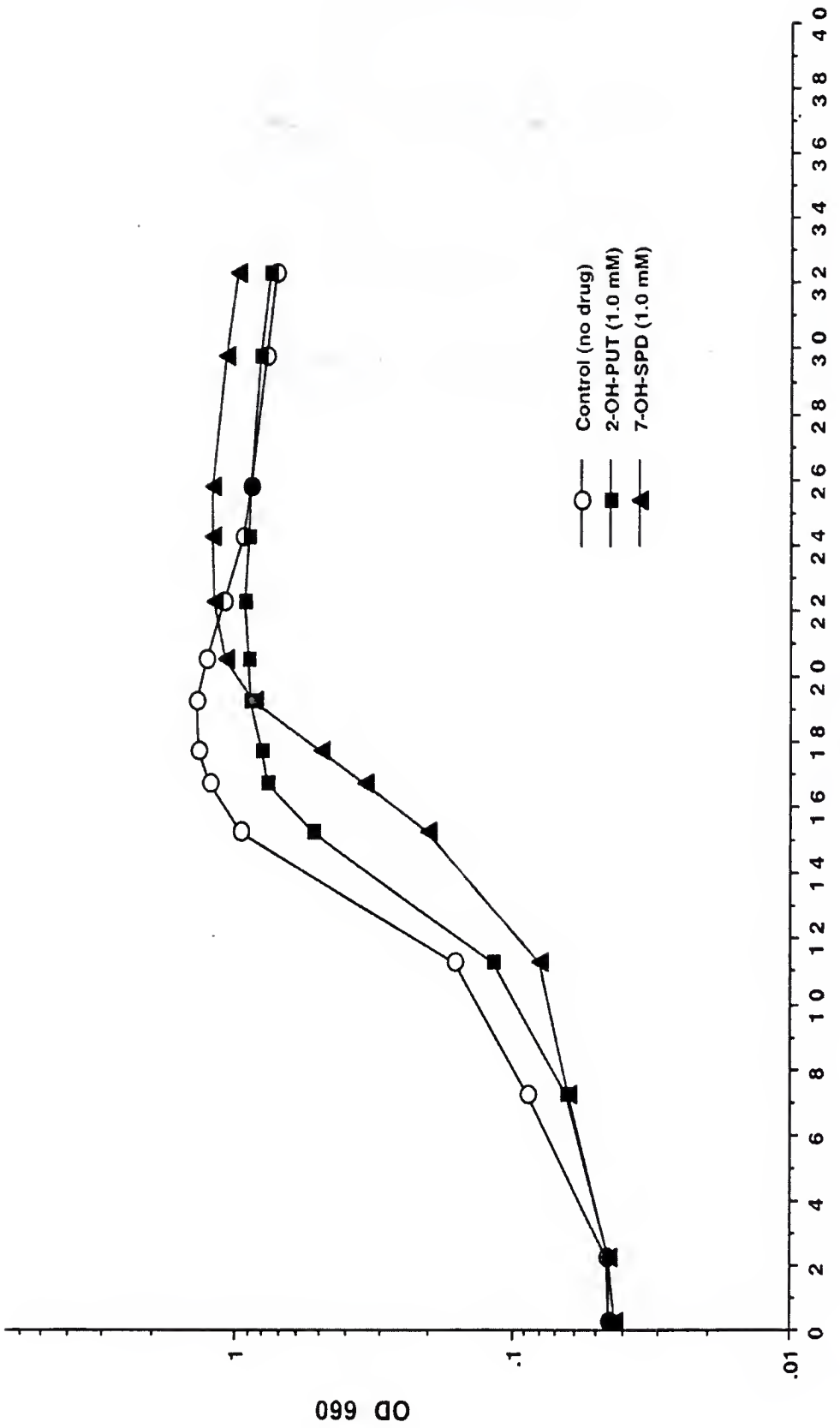


Figure 3-2. Effect of 7-OH-SPD and 2-OH-PUT on the growth of *P. aeruginosa*. Each experiment was performed in duplicate and repeated at least once. Errors in OD660 readings were within 5%.

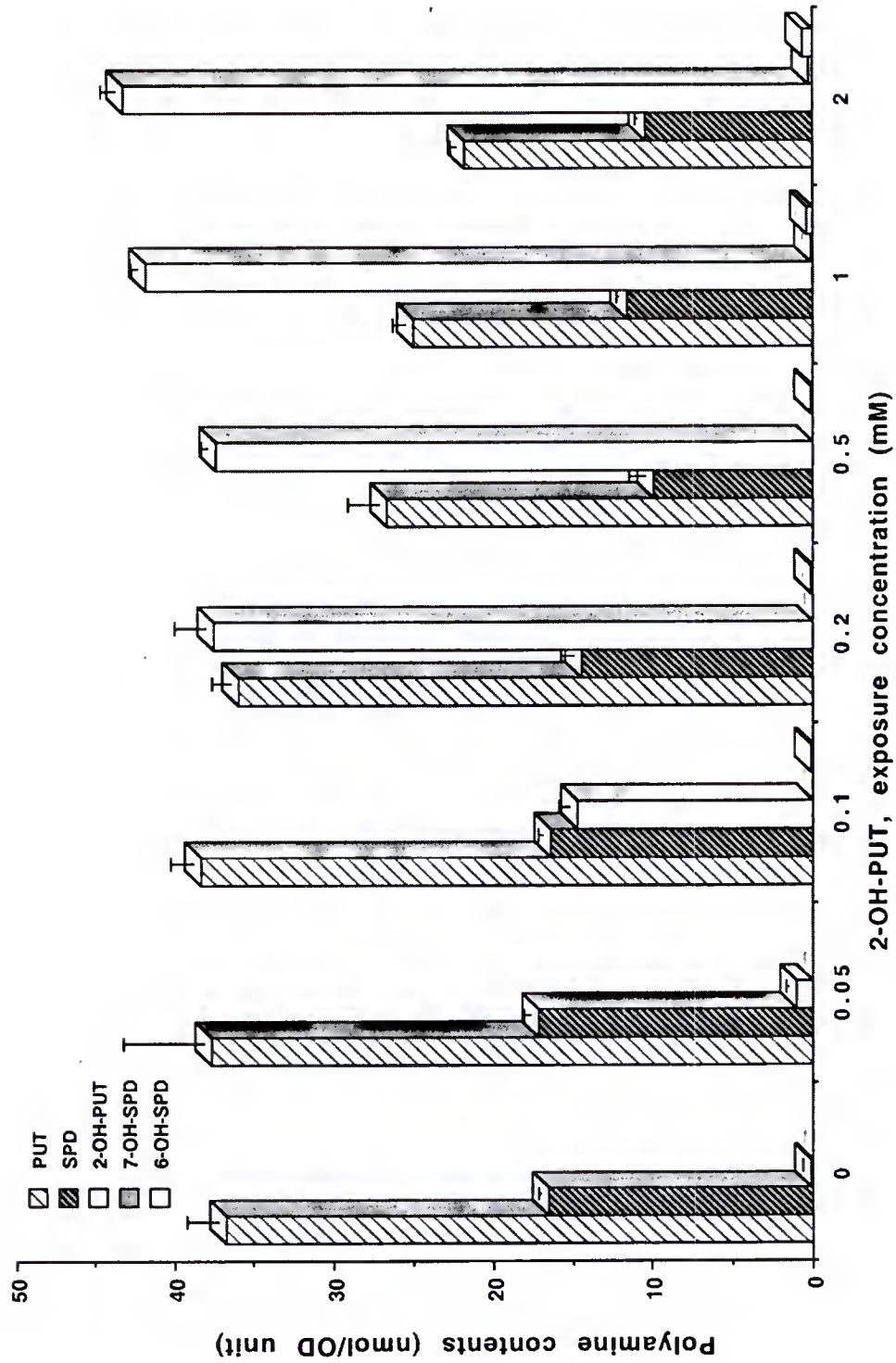


Figure 3-3. Effect of 2-OH-PUT on polyamine contents of *Pseudomonas aeruginosa*.

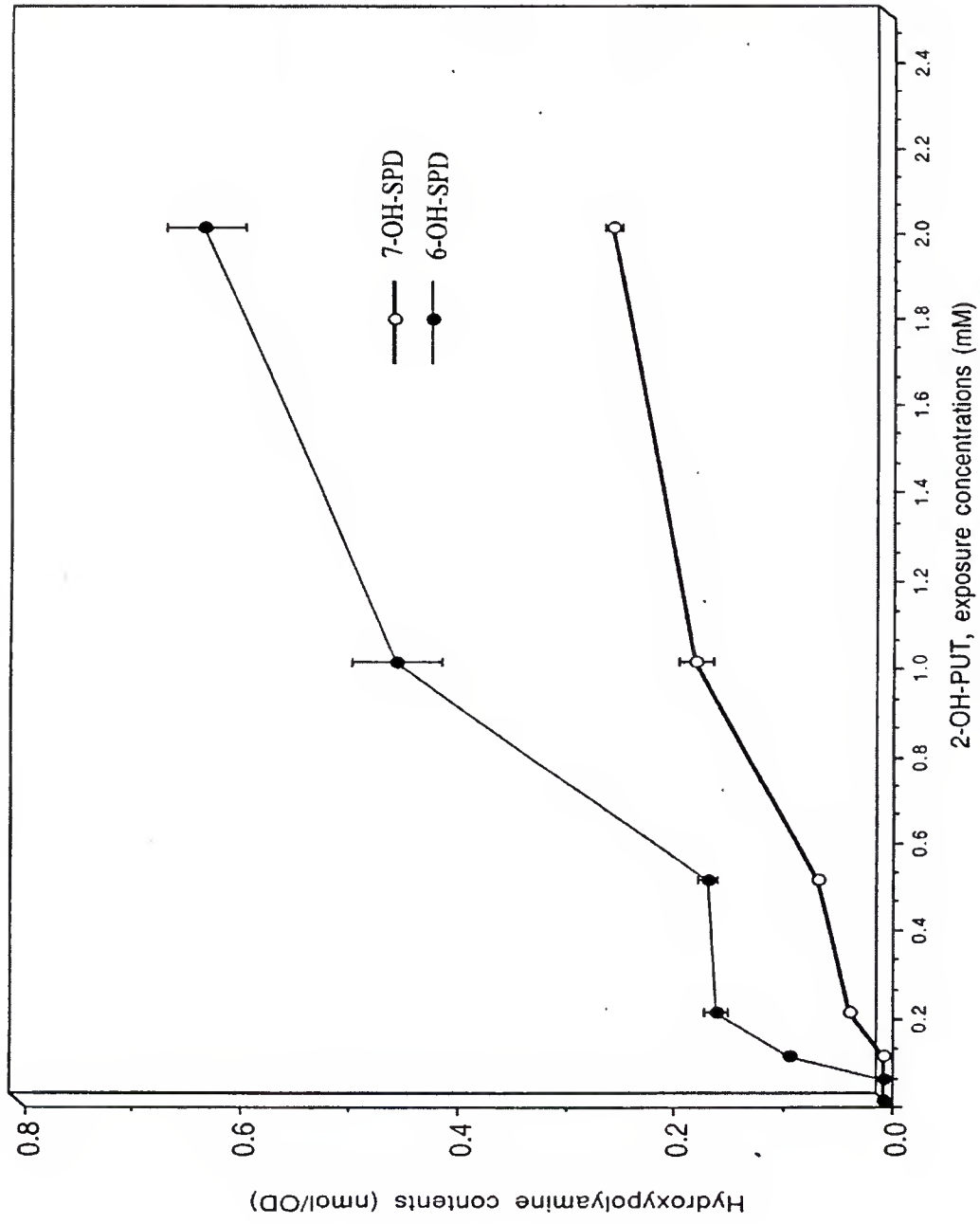


Figure 3-4. Formation of hydroxypolyamines in *P. aeruginosa* upon exposure to 2-OH-PUT

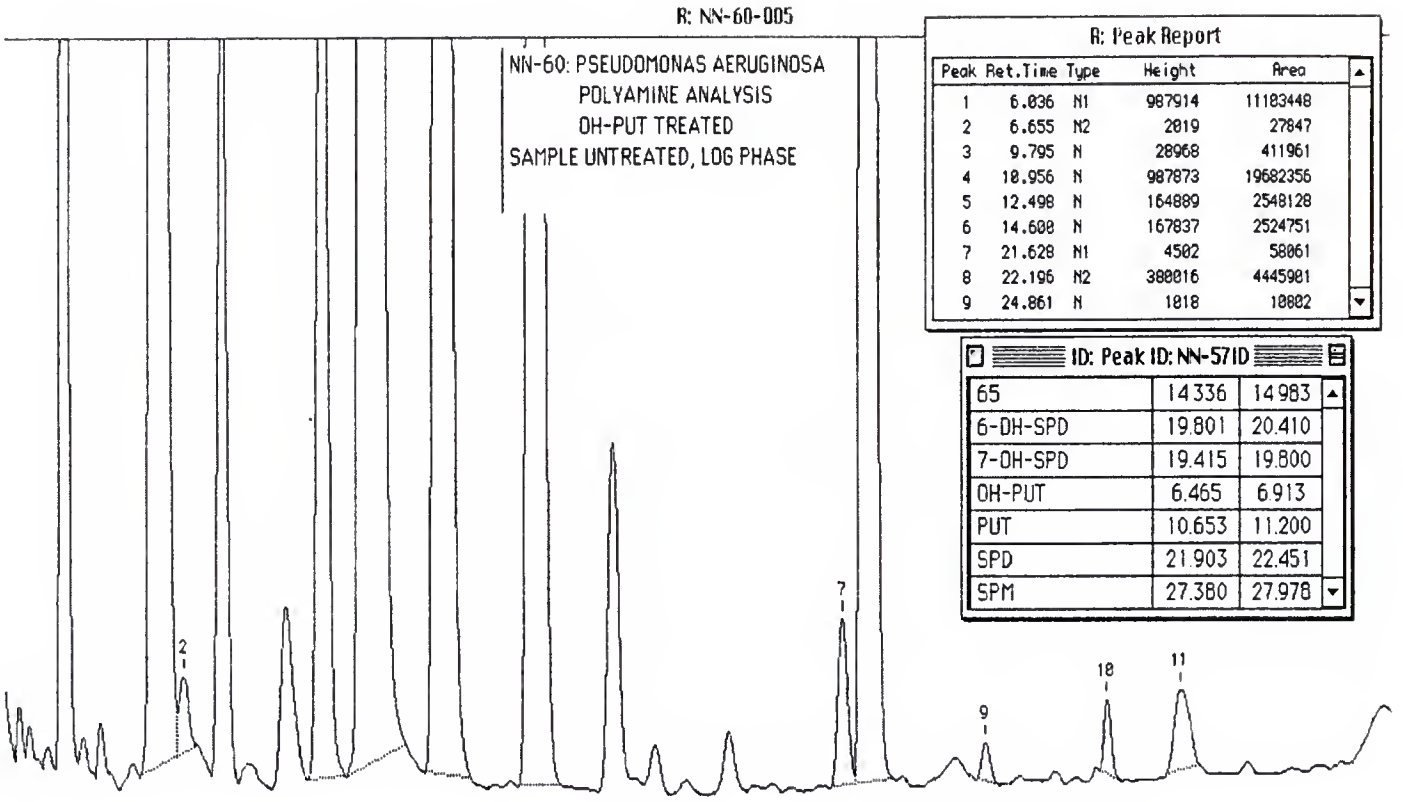


Figure 3-5. HPLC chromatograph of polyamine contents of *P. aeruginosa* (untreated). Bacteria were grown in nutrient broth media without the addition of 2-OH-PUT (also known as OH-PUT) and then harvested at mid log phase for polyamine pool analysis. The peak ID (identification) table describes approximate retention time at which a natural polyamine or its analogue elutes.

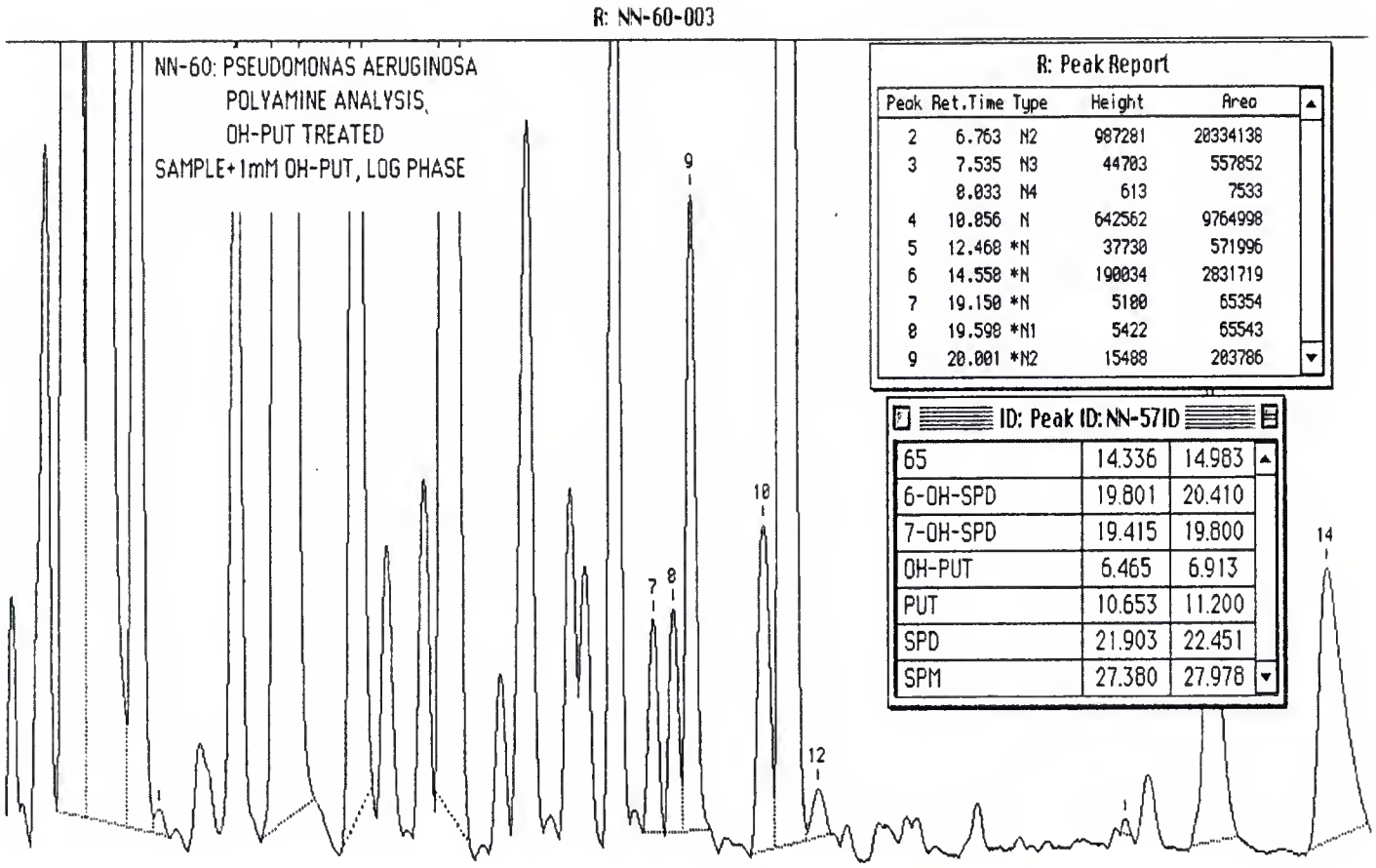


Figure 3-6. HPLC chromatograph of polyamine contents of *P. aeruginosa* grown in the presence of 2-OH-PUT. Bacteria were grown in nutrient broth media with 1.0 mM 2-OH-PUT and then harvested at mid log phase for polyamine pool analysis. In the peak report table, peak # 8 is identified as 7-OH-SPD and # 9 as 6-OH-SPD.

CHAPTER 4
SYNTHESES OF AMINOPOLYAMINES

Design Concept

Based on the surprising observation that *P. aeruginosa* both incorporated and processed 2-OH-PUT and 7-OH-SPD, we elected to further investigate the structural boundary conditions set by this microorganism on the polyamine analogues it would process. We elected to replace the hydroxyl oxygen with another heteroatom, nitrogen. Thus, 2-OH-PUT would be replaced by 2-NH_2-PUT and 7-OH-SPD by 7-NH_2-SPD. This modification could clearly have an important impact on the charge property of the molecule.

Synthesis

Previous Methods

Aminoputrescine (1, 2, 4-butanetriamine, also known as 2-NH_2-PUT) was synthesized in the laboratory as early as 1921 by Windaus and coworkers (Windaus, 1921). It was prepared from histamine by acylation of the imidazole moiety using 2-methylpropanoyl chloride as an acylating agent (Fig. 4-1). The reaction also utilizes aqueous potassium acetate in acetonitrile. This imidazole ring opening reaction, also known as Bamberger ring cleavage, was invented in 1893. Methanolysis of the diformamide isomer mixture **38** yielded a single ene-triamide **39**. The double bond of the ene-triamide intermediate was reduced by hydrogenation in the presence of palladium.

The resulting 1, 2, 4-butane triamide (**40**) was then hydrolyzed by utilizing 6 *N* HCl to furnish 2-NH_2-PUT (**5**).

Recently, Altman and coworkers reported a different method for the Bamberger cleavage of the imidazole ring. Histamine was first acylated at the N-4 position utilizing 2-methylpropanoyl chloride. The imidazole ring of the N^4-(2-methylpropanoyl)histamine was then cleaved in the presence of di-*tert*-butyl dicarbonate and aqueous potassium acetate in acetonitrile (Altman, 1989). Reduction of the ene-triamide was improved by utilizing Raney nickel instead of palladium (Altman, 1991).

In 1993, Altman and Ben-Ishai introduced a new method for synthesizing chiral 2-NH_2-PUT (**37**) (Altman, 1993) (Fig. 4-2). The starting material (*S*)-5-(hydroxymethyl)-pyrrolidone (**30**) was prepared from (*S*)-pyroglutamic acid methyl ester (Silverman, 1980). The alcohol **30** was converted to (*S*)-5-(azidomethyl)-2-pyrrolidone (**31**) by a Mitsunobu reaction utilizing hydrazoic acid and phosphine in diethylazodicarboxylate (DEAD). Hydrogenation of the azide **31** produced the primary amine **32**, which was further hydrolyzed with 6 *N* HCl to afford (*S*)-4,5-diaminovaleric acid (**33**). The diamino acid was acylated with iso-butyl chloroformate to give N^4,N^5-protected amino acid **34,** which was then converted to an azide by the mixed anhydride procedure and sodium azide. Upon heating, this azide intermediate underwent a Curtius rearrangement to form the isocyanate **35**, which was then hydrolyzed by utilizing LiOH in THF and H_2O to yield (*S*)-N^1,N^2-bis(*iso*-butoxycarbonyl)-1,2,4-butanetriamine (**36**). Finally, the di-protected amine **36** was hydrolyzed with 6 *N* HCl to afford (*S*)-1,2,4-butanetriamine (**37**) as a trihydrochloride salt.

In this dissertation, we report a modified procedure for the synthesis of racemic 2-NH_2-PUT. Histamine, without first being acylated at the N-4 position, was subjected to a Bamberger ring cleavage utilizing di-*tert*-butyl dicarbonate. Reduction of the ene-tri-Boc was performed at a higher temperature (55 ^{0}C) to improve the yield. This was a simple method which provided 2-NH_2-PUT in high overall yield (69%). Another advantage of this method was to avoid the azide intermediate of Fig. 4-2, compound **31**, which is known to be explosive and hazardous. The aminopolyamines 7-NH_2-SPD, N^1-ethyl-7-aminospermidine (7-NH_2-MESPD), 7-NH_2-DESPD, and 6-NH_2-SPM were synthesized for the first time in our laboratory. The aminopolyamine 6-NH_2-DESPM was synthesized previously in our laboratory (Bergeron, R. J.; Guangfei, H., unpublished).

Synthesis of 2-NH_2-PUT

The aminopolyamine 2-NH_2-PUT was synthesized in our laboratory according to our modified procedure. Histamine dihydrochloride **1** was subjected to a Bamberger ring cleavage reaction (Bamberger and Liebigs, 1921) in the presence of di-*tert*-butyl dicarbonate (Boc anhydride), aqueous potassium acetate, and acetonitrile stirring at room temperature for 5 d to yield a mixture of *N*-formyl products **2** in 66% yield (Fig. 4-3). Methanolysis of this mixture under refluxing conditions produced a single isomer **3** in 91% yield. At room temperature, the double bond of the ene-tri-boc amine **3** was not completely reduced by hydrogenation with the presence of 10% palladium on activated carbon in absolute ethanol. However, when heated to 55 ^{0}C for 17 h the hydrogenation was completed in 90% yield. Removal of the Boc groups in N^1,N^2,N^4-tri-Boc-1,2,4-butanetriamine (**4**) with 20% $HCl_{(g)}$ in absolute ethanol furnished 2-NH_2-PUT trihydrochloride as a white hygroscopic powder (**5**) in 92% yield.

Synthesis of 7-NH_2-SPD

The two available nitrogens of histamine dihydrochloride **1** were protected by mesitylenesulfonyl (Mts) blocking groups using mesitylenesulfonyl chloride (2.4 equiv) under biphasic conditions (CH_2Cl_2 / 1 *N* NaOH) in 95% yield (Fig. 4-4). The resulting histamine bis(mesitylenesulfonamide) **6** was first treated with NaH in dry DMF and then alkylated at the N-4 position using N-(3-bromopropyl)phthalimide to generate the fully protected N^4-(aminopropyl)histamine **7** in 45% yield. Hydrazinolysis of **7** in refluxing ethanol resulted in the primary amine **8** in 56% yield. The Mts groups in **8** were cleanly removed under strong reductive conditions, using 30% HBr in acetic acid and phenol in CH_2Cl_2, to provide N^4-(3-aminopropyl)histamine trihydrobromide **(9)** in 66% yield. This histamine derivative was then subjected to Bamberger ring opening in the presence of di-*tert*-butyl dicarbonate to afford **10** as a mixture of two isomers in 73% yield. An attempt to cleave the Mts-protecting imidazole in **8** using the same reaction condition failed probably due to steric effects. The formyl groups of the mixture **10** were removed by methanolysis to obtain the ene-tetra-Boc amine **11** in 95%. Reducing the double bond of **11** was achieved successfully by hydrogenation at 55 ^{0}C in the presence of Pd on activated carbon in absolute ethanol to give the tetra-Boc amine **12** in 95% yield. Finally, removal of the Boc groups in **12** by utilizing 20% of $HCl_{(g)}$ in absolute ethanol furnished 7-NH_2-SPD tetrahydrochloride **(13)** as a white hygroscopic solid in 98% yield.

Synthesis of N^1, N^8-Diethyl-7-aminospermidine

A completely different method was developed to prepare 7-NH_2-DESPD. As shown in Fig. 4-5, treating the commercially available N-ethyl-1,3-diaminopropane (**14**) with mesitylenesulfonyl chloride under basic conditions resulted in *N*, *N'*-

bis(mesitylenesulfonyl)-*N*-ethyl-1,3-diaminopropane (**15**) in 80% yield (Bergeron, 2001b). This disulfonamide **15** was alkylated with 4-bromo-1,2-epoxybutane in 90% yield in the presence of NaH and DMF. The alkylation occurred selectively at the bromide end of 4-bromo-1,2-epoxybutane (Cruickshank, 1969). The disulfonamide epoxide **16** was next reacted with N-ethylmesitylenesulfonamide anion (prepared from the corresponding sulfonamide in NaH and DMF), to produce the tri-mesitylene secondary alcohol **17,** in 66% yield. The epoxide opening reaction occurred selectively at the terminal carbon, which was a less sterically hindered electrophile, of **16.** The hydroxyl group of **17** was converted to a corresponding tosylate in 75% yield by reaction with tosyl chloride (1.2 equiv) and pyridine in CH_2Cl_2 at room temperature. The tosylate **18** was reacted with potassium phthalimide in DMF at 85 ^{0}C to afford the phthalimide **19** in 90% yield. It was a surprising result since phthalimide anion was a poor nucleophile and the electrophilic carbon in the tosylate **18** was very sterically hindered. Perhaps, relief of the steric constraint was the driving force for this displacement reaction. Attempting to convert the *N*-phthalimide protecting group in **19** to free amine using hydrazine in refluxing ethanol failed. However, using $NaBH_4$ in 2-propanol and water at room temperature followed by acetic acid at 80 ^{0}C (Osby, 1984) afforded the amine **20** in 55% yield. Finally, the three Mts masking groups in compound **20** were removed under reductive conditions, using 30% HBr in acetic acid and phenol in CH_2Cl_2, and the corresponding polyamine bromide salt was converted to N^1, N^8-diethyl-7-aminospermidine tetrahydrochloride (**21**) in 50% yield.

Synthesis of 6-Aminospermine

The starting material *N*-benzyl-1,3-diaminopropane (**41**), also commercially available, was prepared previously in our laboratory (Bergeron, 1984). Diamine **41** was first treated with mesitylenesulfonyl chloride in CH_2Cl_2 and aqueous NaOH to form the bis(mesitylenesulfonamide) **22** in 60% yield. *N*, *N'*-Bis(mesitylenesulfonyl)-*N*-benzyl-1,3-diaminopropane (**22**) anion, formed in the presence of NaH and DMF, then reacted with 4-bromo-1,2-epoxybutane (Cruickshank, 1969) to afford epoxide **23** in 79% yield (Fig. 4-6). The alcohol **24** was formed regiospecifically, simply due to steric effects, as the sulfonamide anion **22** reacted with epoxide **23** in 65% yield. The hydroxyl group of **24** was converted to a good leaving group, the corresponding *O*-tosyl, in 88% yield, by reaction with tosyl chloride (1.2 equiv) in the presence of pyridine in CH_2Cl_2 at room temperature. The *O*-tosyl in the tosylate **25** was displaced by the phthalimide anion in DMF at 85 ^{0}C to afford protected amine **26** in 54% yield. Conversion of the *N*-phthalimide protecting group in **26** to the free amine was achieved successfully in 63% yield by utilizing $NaBH_4$ in 2-propanol and water at room temperature, followed by treatment with acetic acid at 80 ^{0}C (Osby, 1984). The four Mts masking groups in amine **27** were removed under reductive conditions, using HBr in acetic acid and phenol in CH_2Cl_2 followed by concentrated HCl in ethanol to afford N^1, N^{12}-dibenzyl-6-aminospermine (**28**) as a pentahydrochoride salt. The two benzyl groups in pentaamine **28** were finally removed by hydrogenation at atmospheric pressure in the presence of 1 *N* HCl and 10% palladium on activated carbon. The final racemic product 6-aminospermine (**29**) was generated in 60% yield from compound **27** after two synthetic steps.

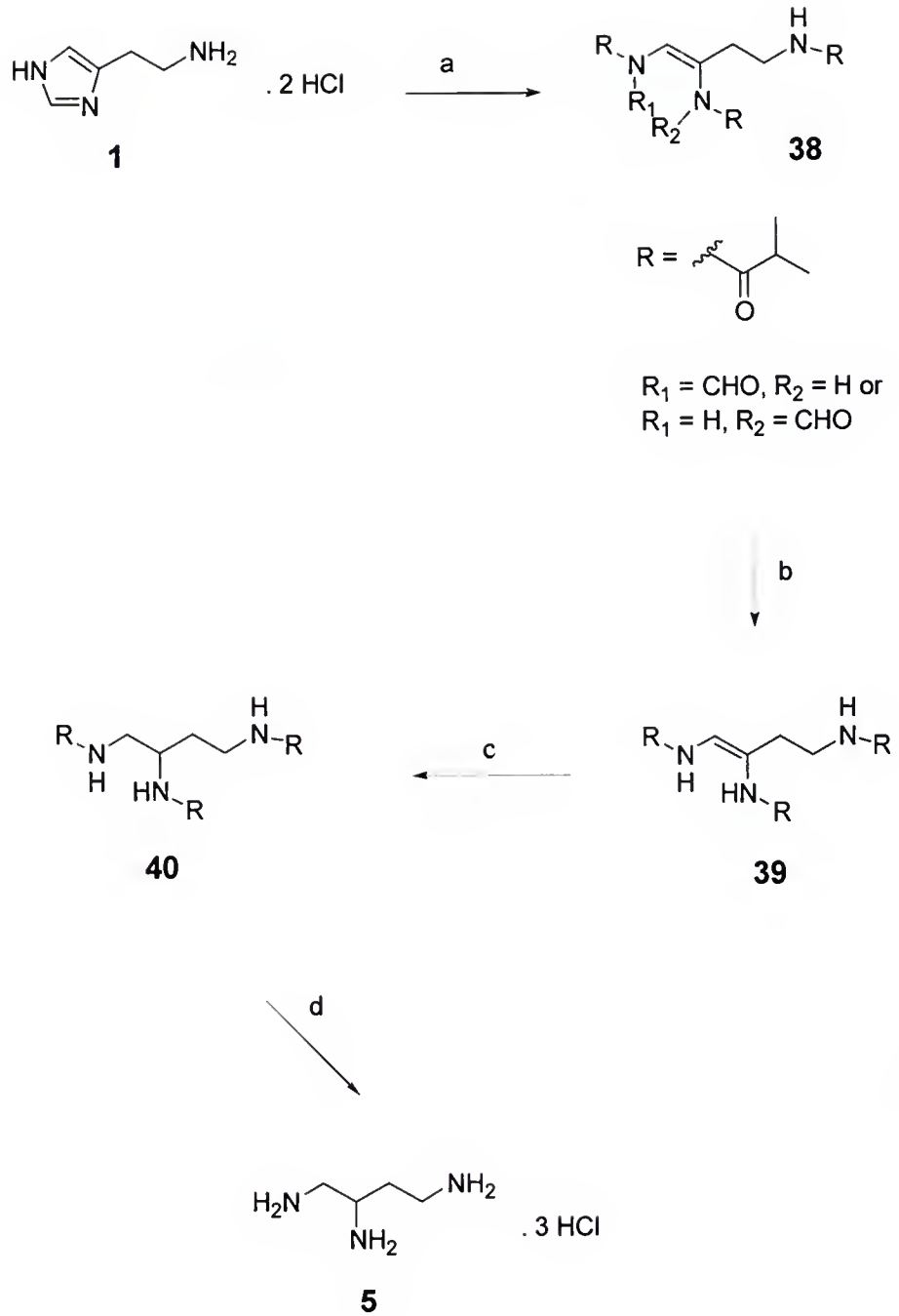


Figure 4-1. A Known Synthetic Route of Racemic 2-NH_2-PUT.
Reagents: (a) RCl/Potassium acetate/H_2O/CH_3CN,
(b) CH_3OH/Reflux, (c) H_2/Pd, (d) 6 *N* HCl

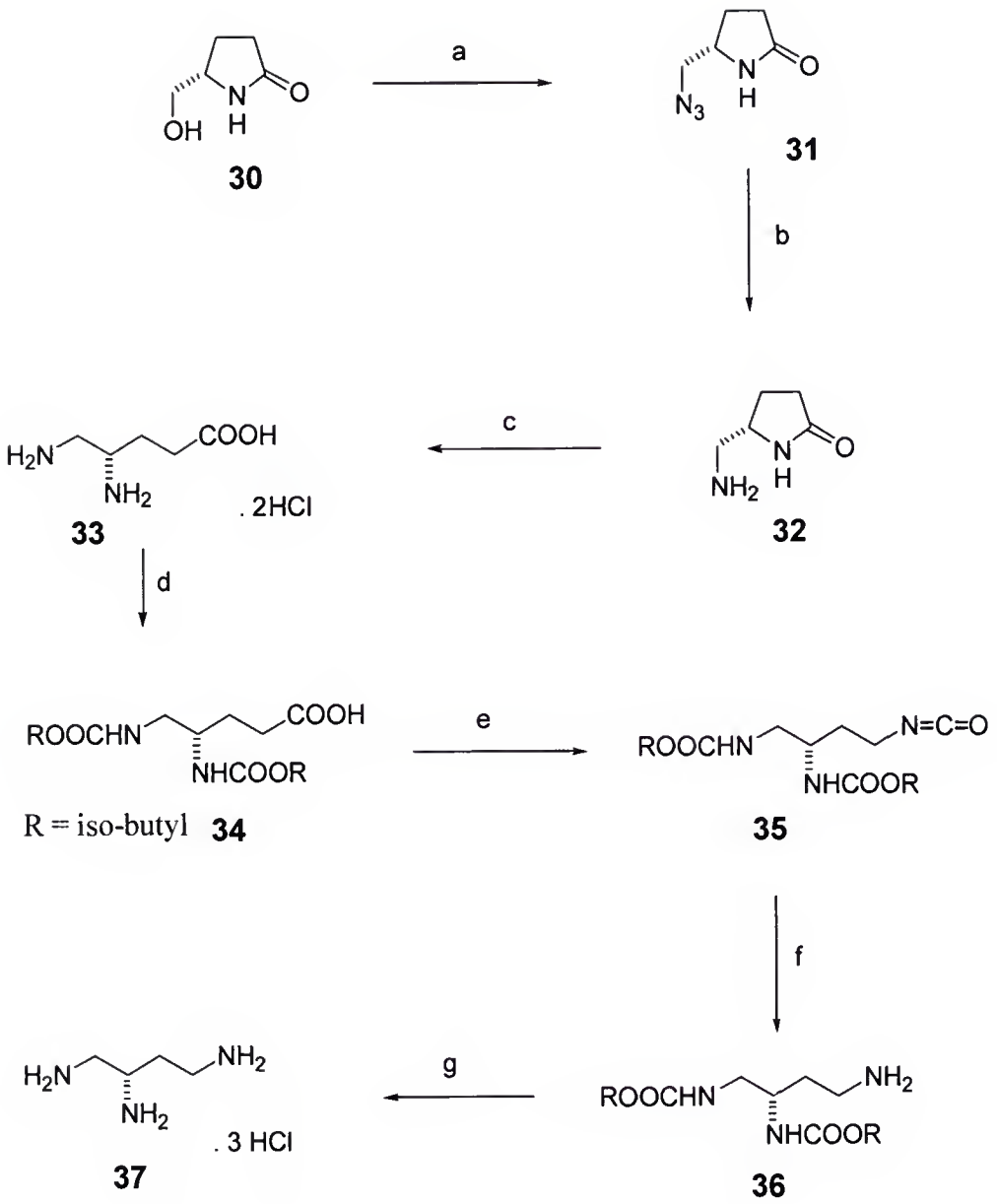


Figure 4-2. A known Synthesic Route of (S)-2-NH_2-PUT.
Reagents: (a) PPh_3-HN_3-DEAD, (b) H_2/Pd, (c) 6 *N* HCl, (d) ClCOOR, (e) ClCOOR, TEA/ NaN_3/ Heat, (f) THF-H_2O/ LiOH, (g) 6 *N* HCl.

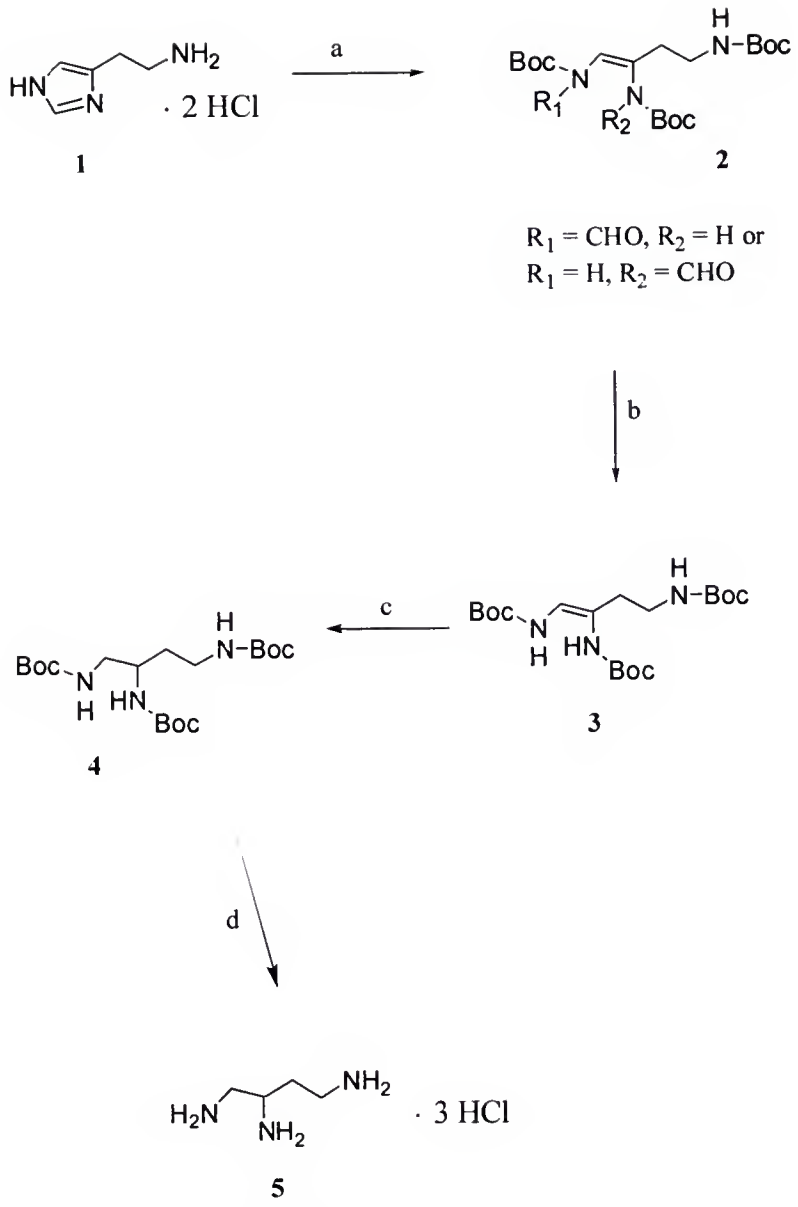


Figure 4-3. Synthesis of 2-aminoputrescine (2-NH_2-PUT)
Reagents: (a) $(Boc)_2O$, KOAc/H_2O/CH_3CN, r.t., 5 d, 66%,
(b) MeOH, reflux, 4 h, 91%, (c) H_2, Pd-C, 55 ^{0}C, 17 h, 90%,
(d) 20% HCl/EtOH, r.t., 12 h, 92%.

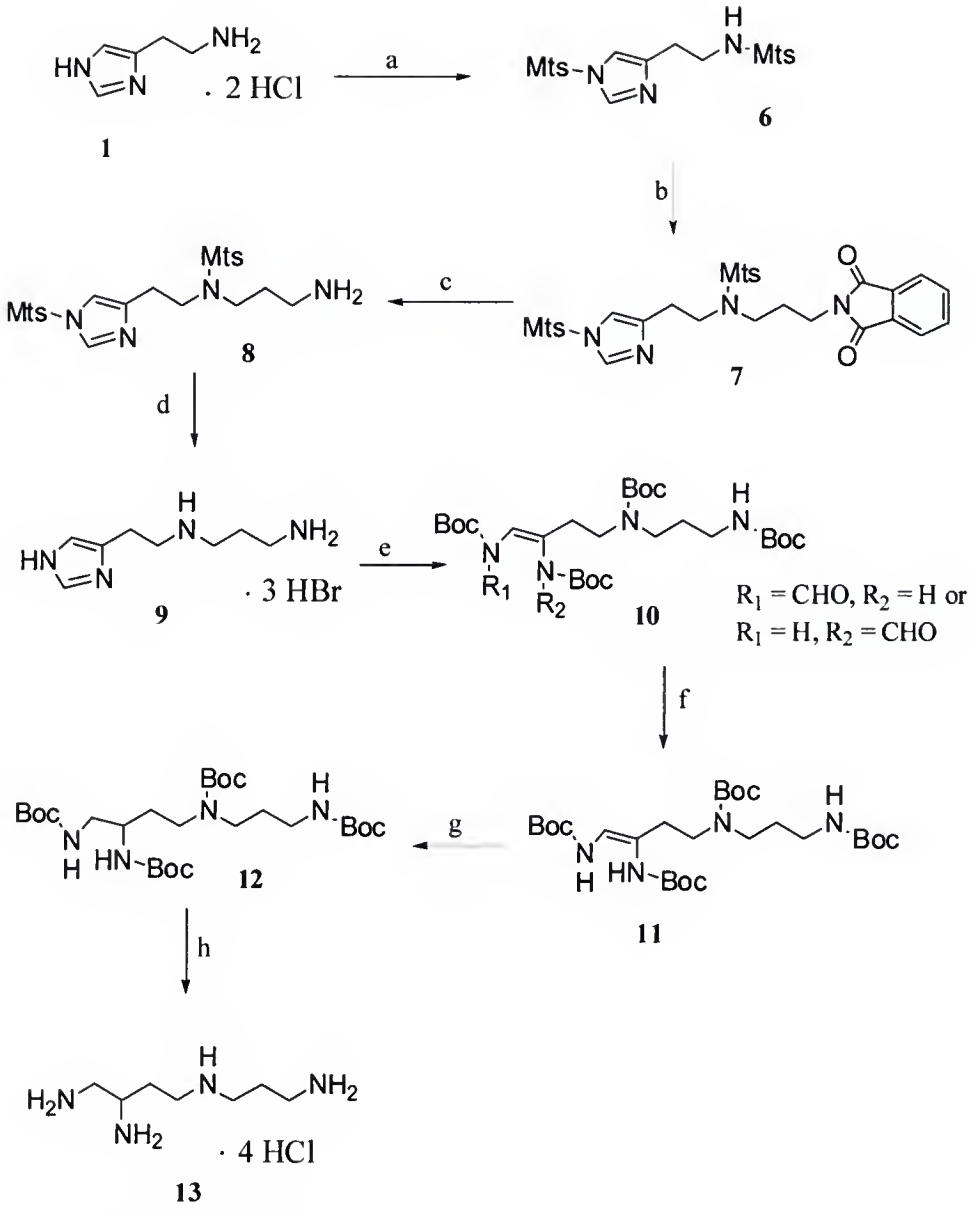


Figure 4-4. Synthesis of 7-aminospermidine (7-NH_2-SPD).
Reagents: (a) MtsCl/CH_2Cl_2, 1 *N* NaOH, 0 ^{0}C to rt, 95%; (b) NaH/DMF, N-(3-bromopropyl)phthalimide, 45%; (c) Hydrazine/EtOH, reflux, 56%; (d) 30% HBr/AcOH, phenol, CH_2Cl_2, 66%; (e) $(Boc)_2O$, KOAc/H_2O, CH_3CN, rt, 73%; (f) MeOH, reflux, 95%; (g) H_2, Pd-C, EtOH, 55 ^{0}C, 12h, 95%; (h) 20% HCl/EtOH, 12h, r.t., 98%.

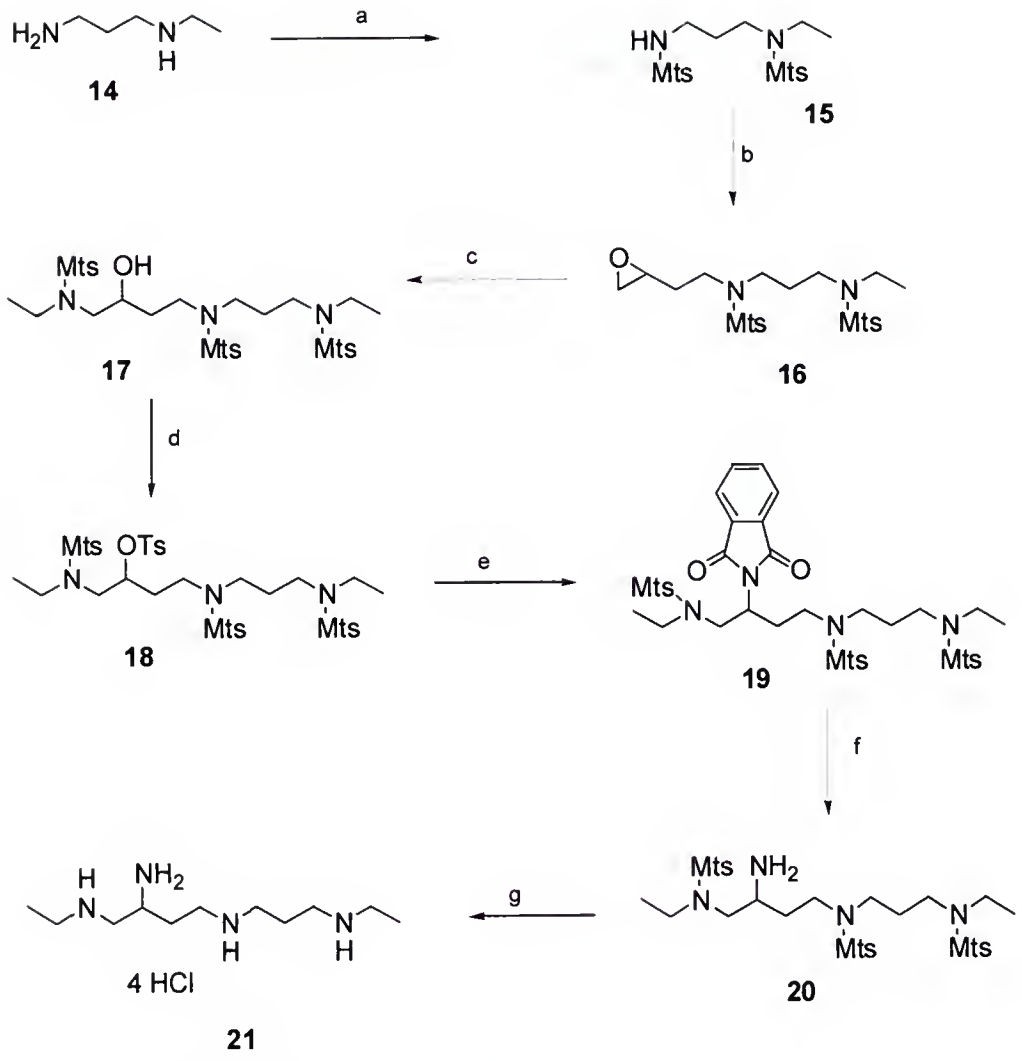


Figure 4-5. Synthesis of N^1,N^8-diethyl-7-aminospermidine

Reagents: (a) MtsCl, CH_2Cl_2, 1 N NaOH, 80%; (b) NaH/DMF, 4-Bromo-1,2-epoxybutane, rt, 90%; (c) NaH/DMF/ CH_3CH_2NH-Mts, 70 ^{0}C, 20 h, 66%; (d) TsCl, CH_2Cl_2, Pyr, rt, 20 h, 75%; (e) Potassium Phthalimide, DMF, 85 ^{0}C, 20 h, 90%; (f) $NaBH_4$/2-propanol/H_2O, rt, 20 h then AcOH, 80 ^{0}C, 20 h, 55%; (g) 30% HBr/AcOH, phenol, CH_2Cl_2, 0 ^{0}C to rt, 20 h, then conc. HCl/EtOH, 50%.

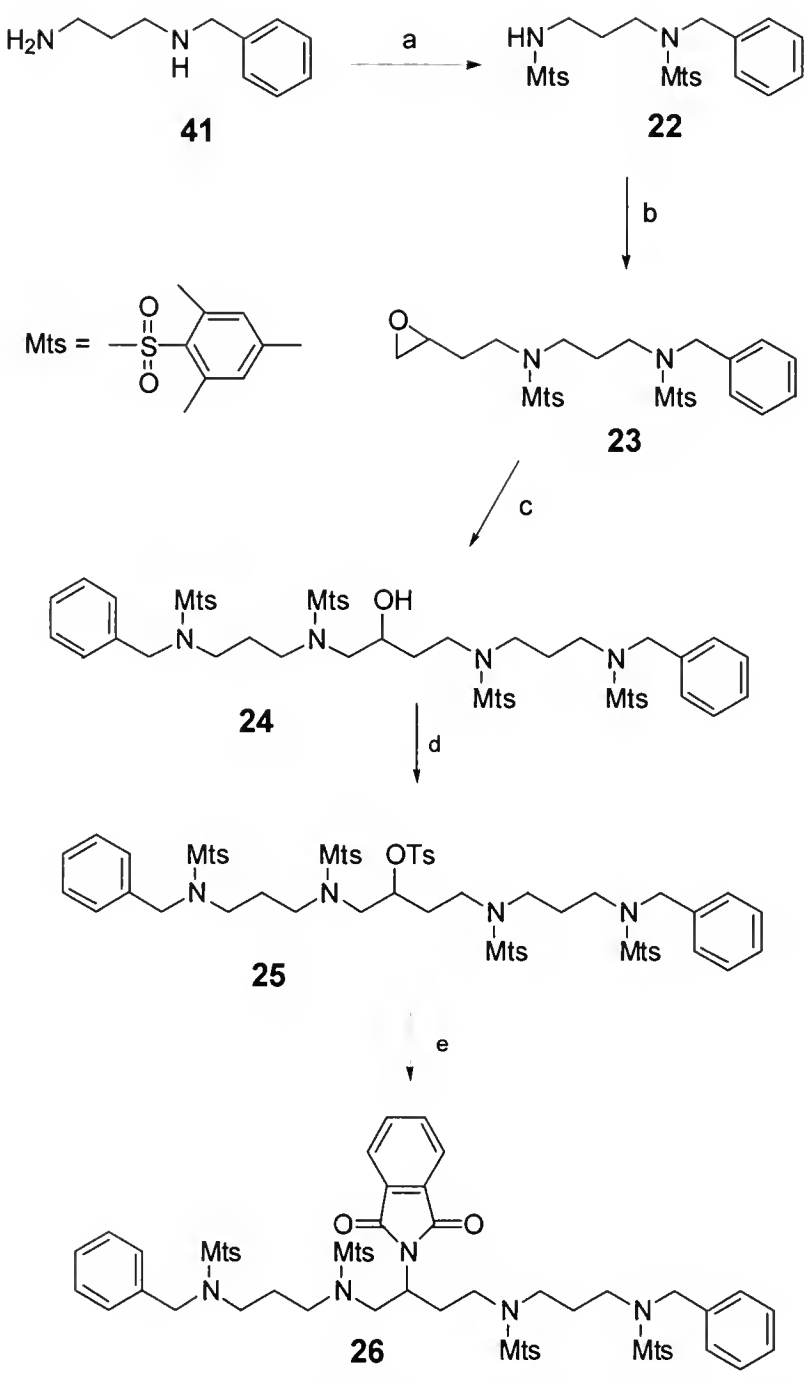


Figure 4-6. Synthesis of 6-aminospermine (continued on next page)

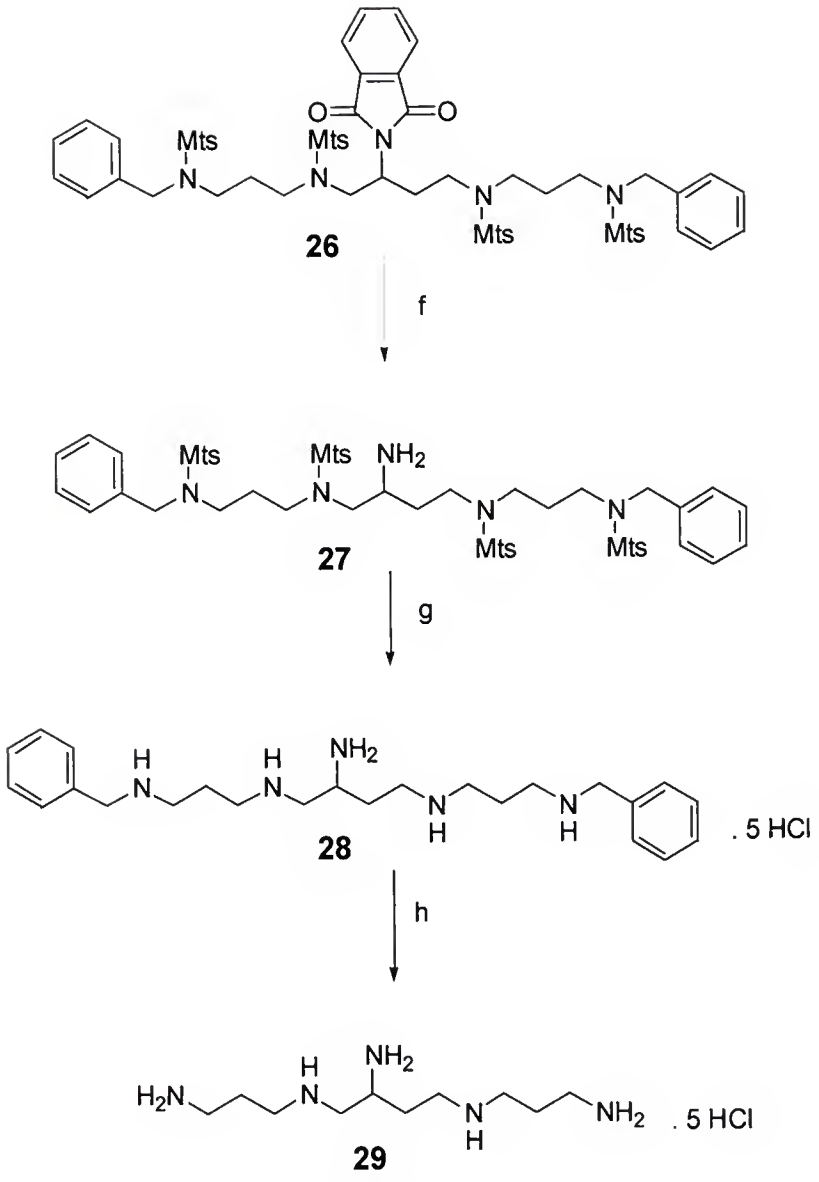


Figure 4-6. Synthesis of 6-aminospermine (continued)

Reagents: (a) MtsCl/ NaOH/ CH_2Cl_2, 60%; (b) NaH/ DMF/ rt, then 4-bromo-1,2-epoxybutane, 79%; (c) **22**, NaH/ DMF/ 85 ^{0}C, 65%; (d) TsCl/ Pyr/ CH_2Cl_2, 88%; (e) Potassium phthalimide/ DMF/ 90 ^{0}C, 54%; (f) $NaBH_4/H_2O$/ 2-propanol, then acetic acid, 80 ^{0}C, 63%; (g) HBr/ acetic acid/ Phenol/ CH_2Cl_2, then conc. HCl; (h) H_2/ 10% Pd-C/ 55 ^{0}C, 60% from **27**.

CHAPTER 5
BIOLOGICAL ACTIVITIES OF AMINOPOLYAMINES IN *P. AERUGINOSA* AND L1210 CELLS: RESULTS AND DISCUSSION

Microbial Studies

While a great deal of information is known about the metabolism of polyamine analogues in various eukaryotes, little is known how these analogues are managed in prokaryotes. In this study, Pseudomonas aeruginosa was used as a prokaryotic model for the evaluation of the impact of some aminopolyamines on the bacterial polyamine metabolism. The impacts on bacterial polyamine contents and the processing of aminopolyamine by the bacteria were assessed. In addition, the effects of each aminopolyamine on bacterial growth were also measured.

Impact on Bacterial Growth

Bacterial turbidity was monitored by measuring optical density at a wavelength of 660 nm (OD660). Since OD660 only reflected the bacterial protein levels but not the viability, colony forming units (CFU) were also determined.

Bacterial turbidity (OD_{660})

Various aminopolyamines and hydroxypolyamines were tested for their impacts on bacterial growth. In the control experiment, bacteria were grown in nutrient broth without addition of any drug. The bacterial turbidity was determined by optical density at 660 nm (OD_{660}). At a high concentration of 1.0 mM, similar to 2-OH-PUT and 7-OH-SPD, 2-NH_2-PUT and 7-NH_2-SPD slightly interfere with the bacterial growth by

stationary phase (22-28 h). In the control growth, the log phase started at 11 h while in the case of 7-OH-SPD and 7-NH_2-SPD, it started at 15 h (Fig. 5-1). Similar growth retardation activities of 7-OH-SPD and 7-NH_2-SPD were observed until late log phase (t = 19 h) when the growth curves started to diverge. By stationary phase (t = 24 h), 7-OH-SPD reduced the bacterial growth to about 92% ($OD_{660,\ 7\text{-OH-SPD}}$ = 1.2 versus $OD_{660,\ control}$ = 1.3) while 7-NH_2-SPD reduced the bacterial growth to about 73% ($OD_{660,\ 7\text{-NH2-SPD}}$ = 0.95 versus $OD_{660,\ control}$ = 1.3). Since 7-NH_2-SPD showed a slightly better inhibitory effect on bacterial growth, we decided to examine further the activity of this aminopolyamine in terms of dose dependence. At the concentration range of 0.05-0.2 mM, 7-NH_2-SPD had no significant impact on the lag time of the bacterial growth curves. However, at the time when the bacteria reached stationary phase, 0.05 mM 7-NH_2-SPD reduced the bacterial growth to about 93%, 0.1 mM reduced the growth to 85%, and 0.2 mM reduced the growth to 80% of control. At high concentrations such as 0.5 mM and 1.0 mM, this aminopolyamine reduced the bacterial growth to as low as 70% of control. As the exposure concentration of 7-NH_2-SPD increased, the lag time (the amount of time the bacteria took to reach logarithmic phase) also increased (Fig. 5-2). In this particular experiment, the untreated bacteria had a lag time of 8 h while 0.5 mM 7-NH_2-SPD increased the lag time to about 11 h and 1.0 mM increased it to 14 h.

Different concentrations (0.1 mM to 1.0 mM) of each of the two diethyl analogues, 7-NH_2-DESPD and 6-NH_2-DESPM, were also tested for their ability to interfere with bacterial growth. Even at high concentrations, both of these terminally diethylated aminopolyamines had no significant impact in reducing bacterial growth by

stationary phase (22-28 h). However, at a concentration of at least 0.5 mM, there was a 4-6 hour delay before the logarithmic phase started (Fig. 5-3, 5-4).

Colony forming unit (CFU)

Since the OD_{660} does not reflect the amount of viable bacteria, we would like to look at the CFU. Colony forming units were determined during the course of growing *P. aeruginosa* in the presence or absence of drugs (aminopolyamines). At each time point, besides recording OD_{660}, bacterial counts were also performed by serial dilution of the bacterial culture to 10^{-7}, 10^{-8}, and 10^{-9} and spreading these diluted culture solutions on nutrient agar plates. Similar to OD_{660} measurements, at a high concentration of 1.0 mM, 6-NH_2-SPM, 7-NH_2-DESPD, and 6-NH_2-DESPM individually had no significant impact on bacterial growth (Fig. 5-5). However, at a concentration of 1.0 mM, 7-NH_2-SPD was able to reduce the CFU by two logarithmic units (Fig. 5-5). It was interesting that in the presence of 7-NH_2-SPD (1.0 mM), no significant retardation in CFU occurred until shortly after the bacteria reached stationary phase (t = 19 h). In the corresponding OD_{660} growth curve (Fig. 5-6), 7-NH_2-SPD (1.0 mM) also showed the greatest impact on bacterial growth although it was a moderate effect. The result from OD_{660} growth studies were consistent with CFU studies both of which indicated that 7-NH_2-DESPD, 6-NH_2-SPM, and 6-NH_2-DESPM had no significant impact on bacterial growth (by stationary phase). Since 7-NH_2-SPD at 1.0 mM had some significant effects on the bacterial cell division, we further investigated the inhibitory activity of this aminopolyamine. Bacteria were incubated with 7-NH_2-SPD at various concentrations (0.01-0.5 mM) and CFU was performed during the course of bacterial growing (Fig. 5-7). At concentrations of 0.01 mM and 0.1 mM, 7-NH_2-SPD showed unnoticeable impact on bacterial CFU. However,

at 0.5 mM, a reduction of CFU by one logarithmic unit was observed. Thus, at a concentration of at least 0.5 mM, 7-NH_2-SPD significantly reduced the cell division ability of *P. aeruginosa* shortly after the bacteria reached stationary phase. The impact of each aminopolyamine on polyamine metabolism of *P. aeruginosa* is more interesting as discussed in the next section.

Processing of Aminopolyamines by *P. aeruginosa*

As shown in Fig. 5-8, when *P. aeruginosa* was exposed to 7-NH_2-SPD at a concentration of 1.0 mM and harvested at early log phase (15-16 h), the intracellular level of 7-NH_2-SPD was three times that of 2-NH_2-PUT. When harvested at mid log phase (19-20 h) or stationary phase (26 h), 7-NH_2-SPD level was reduced to 75% of the 2-NH_2-PUT level. However, in a separate experiment, the bacteria was grown in various concentrations (0.05 – 0.5 mM) of 7-NH_2-SPD and harvested at stationary phase, the intracellular 7-NH_2-SPD was observed to be 33% of the intracellular 2-NH_2-PUT (Fig. 5-9). Thus, the conversion of 7-NH_2-SPD to 2-NH_2-PUT in *P. aeruginosa* exposed to 7-NH_2-SPD was not completed until the bacteria reached stationary phase. In the control untreated bacteria, PUT and SPD levels were 15-20 nmole/OD unit and 4-6 nmole/OD unit, respectively but when exposed to 0.05 mM of 7-NH_2-SPD, these natural polyamine levels reduced to 5-7 nmole/OD unit and < 1 nmole/OD unit, respectively while the intracellular 2-NH_2-PUT and 7-NH_2-SPD levels were 3-4 nmole/OD unit and < 1 nmole/OD unit, respectively. At an exposure concentration of 0.2 mM, the intracellular levels of PUT and SPD were further reduced to < 2 nmole/OD unit. At 0.5 mM, these natural polyamines were reduced to < 1 nmole/OD unit while 2-NH_2-PUT and 7-NH_2-SPD continued to increase to 45-50 nmole/OD unit and 14-16 nmole/OD unit,

respectively. It appeared that at the exposure concentration range of 0.05- 0.5 mM, conversion from 7-NH_2-SPD to 2-NH_2-PUT occurred to an extent that resulted in 2-NH_2-PUT and 7-NH_2-SPD at a 3:1 molar ratio. On the other hand, it is interesting to note that at a concentration of 1.0 mM, a large amount of 7-NH_2-SPD still remained unprocessed in the bacteria (Fig 5-8, growth phases 2 and 3 on horizontal axis). An investigation was then carried out to study how *P. aeruginosa* process this aminopolyamine in a timely manner. An experiment was performed in which the bacteria were exposed to 1.0 mM 7-NH_2-SPD and analyzed for polyamine contents at different time points. At 10 h, which is the beginning of the logarithmic phase, the intracellular levels of 7-NH_2-SPD and 2-NH_2-PUT were about 65 and 25 nmole/OD unit, respectively while the levels of SPD and PUT were reduced to 2 and 8 nmole/OD unit, respectively (Fig. 5-10). The conversion from 7-NH_2-SPD to 2-NH_2-PUT continued to proceed as a function of time. As time progressed, the levels of 2-NH_2-PUT increased, while 7-NH_2-SPD, PUT, and SPD decreased. When bacteria reached mid log phase (t = 14 h), the intracellular levels of 7-NH_2-SPD decreased to about 40 nmole/OD unit while 2-NH_2-PUT increased to about 55 nmole/OD unit. These results clearly revealed that there was a catabolic conversion from 7-NH_2-SPD to 2-NH_2-PUT, and this conversion plateaued as the bacteria progressed through stationary phase. At 26 h (the end of the stationary phase), the intracellular levels of 7-NH_2-SPD reduced to about 34 nmole/OD unit and 2-NH_2-PUT increased to about 64 nmole/OD unit while SPD and PUT were barely detectable. Note that at high exposure concentration, i.e., 1.0 mM of 7-NH_2-SPD, the bacteria were not able to further convert 7-NH_2-SPD to 2-NH_2-PUT. Besides, the intra-bacterial levels of PUT and SPD were almost completely depleted. The reduction in CFU counts was also observed at

these time points as discussed earlier. Perhaps the complete depletion of intracellular PUT and SPD prevented the bacteria from normal activities such as cell division. Unless specified, *P. aeruginosa* in all experiments were harvested at stationary phase.

In *P. aeruginosa* exposed to 7-NH_2-SPD, the intracellular levels of 2-NH_2-PUT and 7-NH_2-SPD increased while the endogenous levels of PUT and SPD decreased in a dose-dependent manner (Fig. 5-9). When exposed to 7- NH_2-SPD at a concentration of 0.5 mM, a nearly complete depletion of PUT and SPD was observed and these bacterial natural polyamines were replaced by 2-NH_2-PUT and 7-NH_2-SPD at a 3:1 molar ratio. These results indicated that 7-NH_2-SPD was subjected to the SSAT/PAO degradation process taking place within *P. aeruginosa.* The polyamine catabolic enzyme SSAT must have first transferred an acetyl group from acetylCoA to the amine of the aminopropyl moiety of 7-NH_2-SPD. The resulting N^1-acetyl-7-NH_2-SPD was then oxidized by PAO and subsequently hydrolyzed to 2-NH_2-PUT plus 3-acetamidopropanal. In a separate experiment when *P. aeruginosa* was exposed to 2-OH-PUT, elaboration into 7-OH-SPD occurred, although only a relatively small amount of 7-OH-SPD was measured by HPLC. A similar scenario occurred with 2-NH_2-PUT which was elaborated into a small amount of 7-NH_2-SPD in *P. aeruginosa*. Poor substrate affinity for spermidine synthase of 2-OH-PUT and 2-NH_2-PUT may account for these observations. In other words, the bacteria polyamine biosynthetic enzyme SPD synthase does not effectively utilize the two substrates 2-OH-PUT and 2-NH_2-PUT to biosynthesize the corresponding hydroxyspermidine and aminospermidines. When *P. aeruginosa* was exposed to 7-NH_2-SPD, one would anticipate the absence or a very small amount of 6-NH_2-SPM in the bacterial intracellular polyamine pool since the bacteria do not have spermine

synthase. As expected, polyamine pool analysis showed no formation of 6-NH_2-SPM from 7-NH_2-SPD in *P. aeruginosa*. In a separate experiment when the bacteria were grown in the presence of 6-NH_2-SPM, significant levels of 7-NH_2-SPD and 2-NH_2-PUT were detected. It is interesting that *P. aeruginosa* was not able to biosynthesize 6-NH_2-SPM from 7-NH_2-SPD, but it was able to utilize 6-NH_2-SPM and catabolically process this exogenous aminopolyamine, likely via the SSAT/PAO pathway, to produce 7-NH_2-SPD and 2-NH_2-PUT.

Expected polyamine catabolism processes such as deethylation and SSAT/PAO pathway have occurred in *P. aeruginosa* when the bacteria were exposed to either 7-NH_2-DESPD or 6-NH_2-DESPM (Fig. 5-11). Common metabolites, including N^1-ethyl-7-aminospermidine (7-NH_2-MESPD), 7-NH_2-SPD, and 2-NH_2-PUT, were detected by HPLC analyses.

Impact on the Total Intracellular Charges in *P. aeruginosa*

Previous studies revealed that there was a conservation of charge with respect to total amine equivalence in the cells treated with various spermidine and spermine analogues (Bergeron, 1997). After 48 h of exposure to an aminopolyamine, the total intracellular charge was calculated by adding the charges of the natural polyamines, the aminopolyamine and its metabolites. For example, each equivalent of PUT is associated with two equivalents of cationic charges, each equivalent of SPD with three, and each equivalent of SPM with four. Each aminopolyamine has an additional amino group compared to its parent natural polyamine. Thus, if all nitrogen atoms in amino-polyamines are protonated at physiological pH, each equivalent of 2-NH_2-PUT will be associated with three equivalents of cationic charge, each equivalent of 7-NH_2-SPD or

7-NH_2-DESPD with four, and each equivalent of 6-NH_2-SPM or 6-NH_2-DESPM with five.

The total intracellular charge was not conserved in *P. aeruginosa* when the bacteria were exposed to each aminopolyamine. In the untreated bacteria, the total charge was about 112.24 $\pm$ 15.36 nanoequiv/OD unit while in the treated bacteria, the total charge increased significantly (Table 5-1). The larger charge amounts (Table 5-1, middle column) were calculated based on the assumption that all nitrogen atoms of the aminopolyamines were protonated at physiological pH, and the smaller charge amounts (Table 5-1, far right column) were estimated assuming that the last nitrogen of each aminopolyamine was not protonated. Thus, when the bacteria were incubated with 7-NH_2-SPD (1.0 mM) for 22-24 h (stationary phase), the total intracellular charge increased to 169.94-272.87 nanoequivalent/OD unit (1.5-2 fold of control) (Table 5-1). It appeared that the bacteria can tolerate a high amount of charge without disrupting their growth. There was an exception in the case of 7-NH_2-SPD (1.0 mM) in which the bacterial growth was reduced by two logarithmic units at the stationary phase. Our result clearly indicated that the accumulation of charge was not responsible for this growth reduction. This rationale holds true because the bacteria survived without any significant growth reduction when exposed to other aminopolyamines (7-NH_2-DESPD and 6-NH_2-DESPM), at which the total intracellular charge was markedly elevated (Table 5-1).

Eukaryotic Studies

The murine L1210 cell is routinely used to study polyamine metabolism. In this model, a great deal of information is available on the key enzymes involved in the biosynthetic pathway as well as in the processing of polyamine analogues. The effects on

cell growth in addition to the impact on polyamine metabolism of each aminopolyamine were measured. Results from 6-NH_2-DESPM studies (Bergeron, R. J.; Guangfei H.; and Yao, H., unpublished) were compared with the results from studies of other amino-polyamines in this dissertation.

IC_{50} values in L1210 Cells

As shown in Table 5-2, 2-NH_2-PUT, 7-NH_2-DESPD and 6-NH_2-SPM had no significant impact on the growth of L1210 cells. These three aminopolyamines had 48-h IC_{50} values of 100 μM and greater. At 96 h, 7-NH_2-DESPD and 6-NH_2-SPM, both with IC_{50} values of 6 and 10 μM, respectively, were much more active than 2-NH_2-PUT although this effect on cellular growth was still very moderate. At 48 h, 7-NH_2-SPD was almost twice as active as its diethyl analogue, 7-NH_2-DESPD, but at 96 h their activities were similar. The aminopolyamine 6-NH_2-DESPM had a 48-h IC_{50} of 9 μM and a 96-h IC_{50} of 0.35 μM (Bergeron et al, unpublished). Among all aminopolyamines studied (Table 5-2), 6-NH_2-DESPM was the most active analogue in reducing the cell growth.

Competitive Uptake Determination in L1210 Cells

The ability of 2-NH_2-PUT, 7-NH_2-SPD, 6-NH_2-SPM, and 7-NH_2-DESPD to compete with radiolabeled SPD for uptake was evaluated (Table 5-2). As predicted, 2-NH_2-PUT had a K_i value of > 500 μM. The diethyl analogue 7-NH_2-DESPD also had a poor uptake affinity with a K_i value of 130 μM while the free amine parent (7-NH_2-SPD), with a K_i of 84 μM, was a more effective competitor. A similar trend was seen in which 6-NH_2-SPM ($K_i = 21$ μM) was better than 6-NH_2-DESPM ($K_i = 33.7$ μM) (Bergeron et al, unpublished) in competing with SPD for uptake. The general trend is that the terminally diethylated aminopolyamines have higher K_i values than the

unethylated aminopolyamines and are thus less easily taken up by the cells. In addition, the aminospermine analogues have lower K_i values than the aminospermidine analogues (6-NH_2-SPM versus 7-NH_2-SPD; 6-NH_2-DESPM versus 7-NH_2-DESPD). Thus, the aminospermines, namely 6-NH_2-SPM and 6-NH_2-DESPM, are more easily taken up by the cell compared to the aminospermidines, namely 7-NH_2-SPD and 7-NH_2-DESPD.

Bergeron R. J. and coworkers have shown that charge is critical to cellular recognition of the polyamine analogues at the transport level. Factors that influence the "polyamine recognition" by the uptake apparatus are (1) the number of nitrogens in the polyamine analogue, (2) the spacing between the nitrogens, and (3) the steric effects of substituents in proximity to the nitrogens. Studies in Bergeron's laboratory revealed that tetraamines, e.g., N^1,N^{12}-diethylspermine (DESPM) and *N*,*N*'-bis(4-piperidinylmethyl)-1,4-diaminobutane [PIP (4,4,4)], both of which form tetracations at physiological pH, compete well with SPD for uptake (Bergeron, 1995). The K_i values for DESPM and PIP (4,4,4) are 1.6 μM and 4.9 μM, respectively. The triamines, e.g., N^1,N^8-dimethylspermidine (DMSPD) and homospermidine (HSPD), both of which form trications at physiological pH, also compete well with SPD for the polyamine transport apparatus. These two triamines have competive K_i values of 5.1 μM (DMSPD) and 3.4 μM (HSPD). It was concluded that as long as a polyamine analogue has at least +3 charges separated by 3 or 4 methylene groups, it will be "recognized" by the polyamine transport apparatus. On the other hand, dicationic polyamine analogues such as N^1,N^{12}-bis(2,2,2-trifluoroethyl)spermine (FDESPM) (K_i value of 285 μM), or PUT ($K_i > 500$ μM), competes poorly with SPD (Bergeron, 1995).

As mentioned earlier, 2-NH_2-PUT (K_i > 500 μM) had a poor affinity for the polyamine uptake apparatus. Although it is a triamine, 2-NH_2-PUT is more likely present as a dication at physiological pH. Thus, similar to FDESPM and PUT, 2-NH_2-PUT competes poorly with SPD for the polyamine uptake apparatus. The observed increase in K_i values when the aminopolyamines are terminally ethylated (Table 5-2) was probably due to the steric effect of the terminal ethyl groups. The ethyl groups interfere with the interaction between the protonated terminal nitrogen cations and the biological counteranions in the transport apparatus, thus reducing the binding affinity of the polyamine analogue.

Impact on ODC and AdoMetDC Activities

The effect of the polyamine analogues on ODC and AdoMetDC is fairly rapid (Porter, 1987 and Porter, 1990). For example, the induced reduction from DESPM on ODC activity plateaued at 4 h (Porter, 1987), and AdoMetDC at 6 h (Porter, 1990). On the basis of these studies, the impact of aminopolyamines on ODC and AdoMetDC were evaluated at 4 h and 6 h, respectively.

At a concentration of 1 μM, 7-NH_2-SPD reduced ODC activity to 72% of control and the corresponding diethyl analogue, 7-NH_2-DESPD, to 79% (Table 5-3). It appeared that the parent aminospermidine and its diethyl analogue had similar impacts on ODC activity. On the other hand, the diethyl aminospermine analogue, 6-NH_2-DESPM, was significantly more active than the corresponding parent aminospermine, 6-NH_2-SPM, with reduction to 19% versus 52% of control. Both aminospermines, 6-NH_2-SPM and 6-NH_2-DESPM, were more active in reducing ODC activity than the aminospermidines, 7-NH_2-SPD and 7-NH_2-DESPD. A similar trend in reduction of AdoMetDC activity was

observed in which the aminospermines were more active than the aminospermidines (Table 5-3). The aminopolyamine 6-NH_2-DESPM was more active than its parent aminospermine, 6-NH_2-SPM, in reducing AdoMetDC activity. At a concentration of 1 μM, 6-NH_2-DESPM reduced AdoMetDC activity to 53% of control, while 6-NH_2-SPM reduced enzyme activity to 70% of control. The two aminospermidines 7-NH_2-SPD (reducing AdoMetDC activity to 81%) and 7-NH_2-DESPD (reducing AdoMetDC activity to 86%) had similar effects on the reduction of AdoMetDC activity.

Impact on SSAT Activity

At a concentration of 10 μM for 48 h, each of the following aminopolyamines stimulated SSAT activities: 7-NH_2-SPD, 189%; 7-NH_2-DESPD, 303%; 6-NH_2-SPM, 117%; and 6-NH_2-DESPM, 668% of control. It was noticeable that 7-NH_2-DESPD was slightly more active that its free amine parent (7-NH_2-SPD) in up-regulating the SSAT while 6-NH_2-DESPM was noticeably more active than its corresponding parent 6-NH_2-SPM. The general trend observed was that the terminally diethylated aminopolyamines were more effective than their corresponding parent aminopolyamines in stimulating the activity of SSAT.

Impact on Polyamine Pools and Metabolism of L1210 Cells

When the L1210 cells were exposed to 7-NH_2-SPD, it appeared that there was only a small amount of catabolic conversion to 2-NH_2-PUT (29-35 nmol/OD unit) as a result of SSAT/PAO activities. There was a significant new peak (eluted after spermine) in the HPLC chromatograms of the L1210 cells which increased in a dose-dependent manner. This suspicious peak was later shown to correspond to 6-NH_2-SPM, formed by the aminopropylation of 7-NH_2-SPD. While the control cells did not have any of the

aminopolyamines (Fig. 5-12), the cells incubated with 60 μM 7-NH_2-SPD for 48 h had 6-NH_2-SPM with an area under the curve of about 10% of the SPM area (Fig. 5-13). When the cells were incubated with 300 μM 7-NH_2-SPD, 6-NH_2-SPM achieved a concentration of ~ 25-30% of SPM (Fig. 5-14).

When cells were treated with 60 μM of 7-NH_2-SPD for 48 h, intracellular PUT, SPD, and SPM levels decreased to 35%, 34%, and 77% of control levels, respectively (Table 5-4). At the same exposure concentration, the intracellular level of 7-NH_2-SPD was about 2500 pmole/10^6 cell, which was 2.5 times of that of SPD. This indicated an efficient cellular uptake of 7-NH_2-SPD even though 7-NH_2-SPD showed a poor affinity (K_i = 84 μM) for the polyamine transport apparatus (Table 5-2).

When L1210 cells were exposed to 100 μM of 7-NH_2-DESPD for 48 h, a significant level of the metabolite N^1-ethyl-7-aminospermidine (1765 pmol/10^6 cells) was detected; only a relatively small amount of 7-NH_2-SPD (7 pmol/10^6 cells) was present in the intracellular polyamine pool. At a higher exposure concentration (500 μM), the level of N^1-ethyl-7-aminospermidine increased to 2700 pmole/10^6 cells (1.5 fold) and 7-NH_2-SPD to 12 pmole/10^6 cells. The aminopolyamine 7-NH_2-DESPD must have undergone N^8-deethylation to form N^1-ethyl-7-aminospermidine. A second deethylation then occurred on N^1-ethyl-7-aminospermidine to produce 7-NH_2-SPD (Fig. 5-15). At a concentration of 100 μM, 7-NH_2-DESPD reduced intracellular PUT, SPD and SPM in treated cells to 56%, 29% and 60% of control, respectively while at 500 μM of the same exposure analogue, PUT, SPD and SPM levels were reduced to 43%, 18% and 49%, respectively (Table 5-5).

Incubation of L1210 cells with 10 μM or 50 μM of N^1, N^{12}-diethyl-6-aminospermine (6-NH_2-DESPM) for 48 h also resulted in the formation of two metabolites, N^1-ethyl-7-aminospermidine and 7-NH_2-SPD although the intracellular levels of these metabolites were much lower (Bergeron, R. J.; Guangfei, H.; Yao, H., unpublished). In the case of smaller exposure concentration, N^1-ethyl-7-amino-spermidine was detected at a level of 54 pmol/10^6 cells and 7-NH_2-SPD at 21 pmol/10^6 cells. At the higher exposure concentration, these metabolites were found at similar levels (Table 5-6). First, 6-NH_2-DESPM must have undergone N^1-deethylation before any further metabolism could occur. The resulting N^{12}-ethyl-6-aminospermine then underwent deaminopropylation by the SSAT/PAO polyamine degradation pathway to produce N^1-ethyl-7-aminospermidine (Fig. 5-16). Formation of 7-NH_2-SPD was a result of deethylation of the intermediate metabolite N^1-ethyl-7-aminospermidine. At the 48-h IC_{50} concentration of 10 μM, 6-NH_2-DESPM reduced intracellular PUT, SPD, and SPM of treated cells to 39%, 16%, and 54% of control, respectively (Table 5-6) (Bergeron, R. J.; Guangfei, H.; Yao, H., unpublished). At five times the IC_{50} concentration, i.e. 50 μM, 6-NH_2-DESPM reduced intracellular PUT, SPD, and SPM to 26%, 7%, and 32% of control, respectively. These results indicated that 6-NH_2-DESPM had a greater impact on the polyamine pool of the L1210 cells than 7-NH_2-SPD and the terminally ethylated analog, 7-NH_2-DESPD.

Impact on Total Cellular Charges of L1210 Cells

Our results indicated that there was a slight impact on the total cellular charges when L1210 cells were exposed to each aminopolyamine (Table 5-7). The total charge in untreated cells was 12.78 x 10^3 picoequivalent/10^6 cells. When exposed to 7-NH_2-SPD

(60 μM) for 48 h, the total charge increased to the range $13.43 \times 10^3 \pm 16.00 \times 10^3$ picoequivalent/10^6 cells. As the exposure concentration of 7-NH_2-SPD raised five times to 300 μM, the total intracellular charge slightly increased to $14.79 \times 10^3 \pm 18.45 \times 10^3$ picoequivalent/10^6 cells (1.2 – 1.5 fold of control values). Similar results were recorded when cells were exposed to 6-NH_2-DESPM, which caused the total cellular charge to increase to 1.2 – 1.6 fold of control. In the case of 7-NH_2-DESPD (at two different exposure concentrations, 100 μM and 500 μM, for 48 h), the total cellular charge was conserved. In order to maintain the balance of charge, the cell processed some portions of the natural polyamines and exported them as it incorporated the aminopolyamine. It is interesting to note that, when exposed to 7-NH_2-DESPD, cells did not incorporate this exogenous polyamine analogue beyond the point where the total cellular charge would be disrupted.

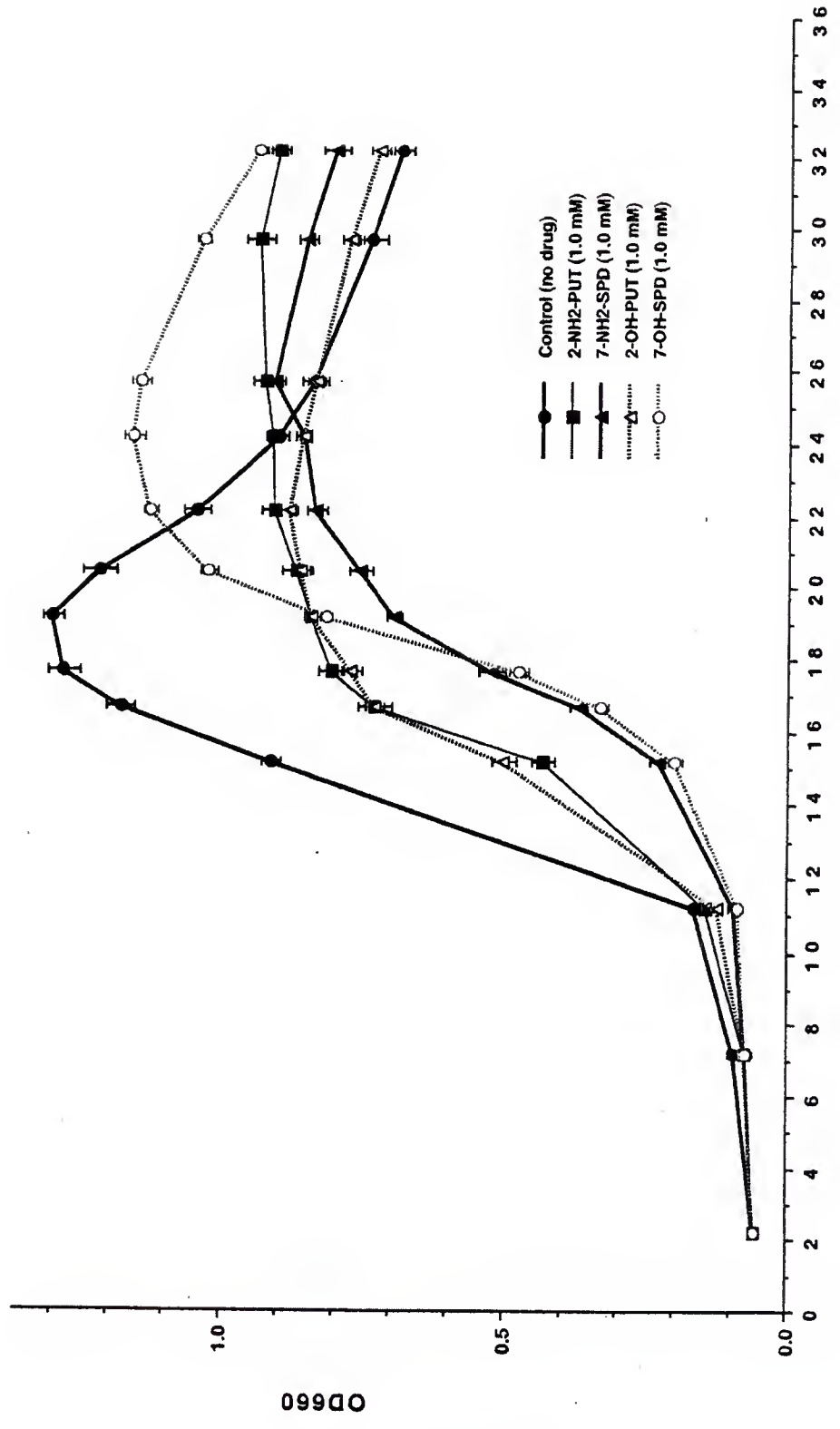


Figure 5-1. Effects of aminopolyamines and hydroxypolyamines on *P. aeruginosa* growth. The bacteria were exposed to 2-NH_2-PUT, 7-NH_2-SPD, 2-OH-PUT, and 7-OH-SPD (1.0 mM each). The turbidity (OD_{660}) was plotted against time (h). Data are average values from a single experiment performed in duplicate. Bars indicated ranges.

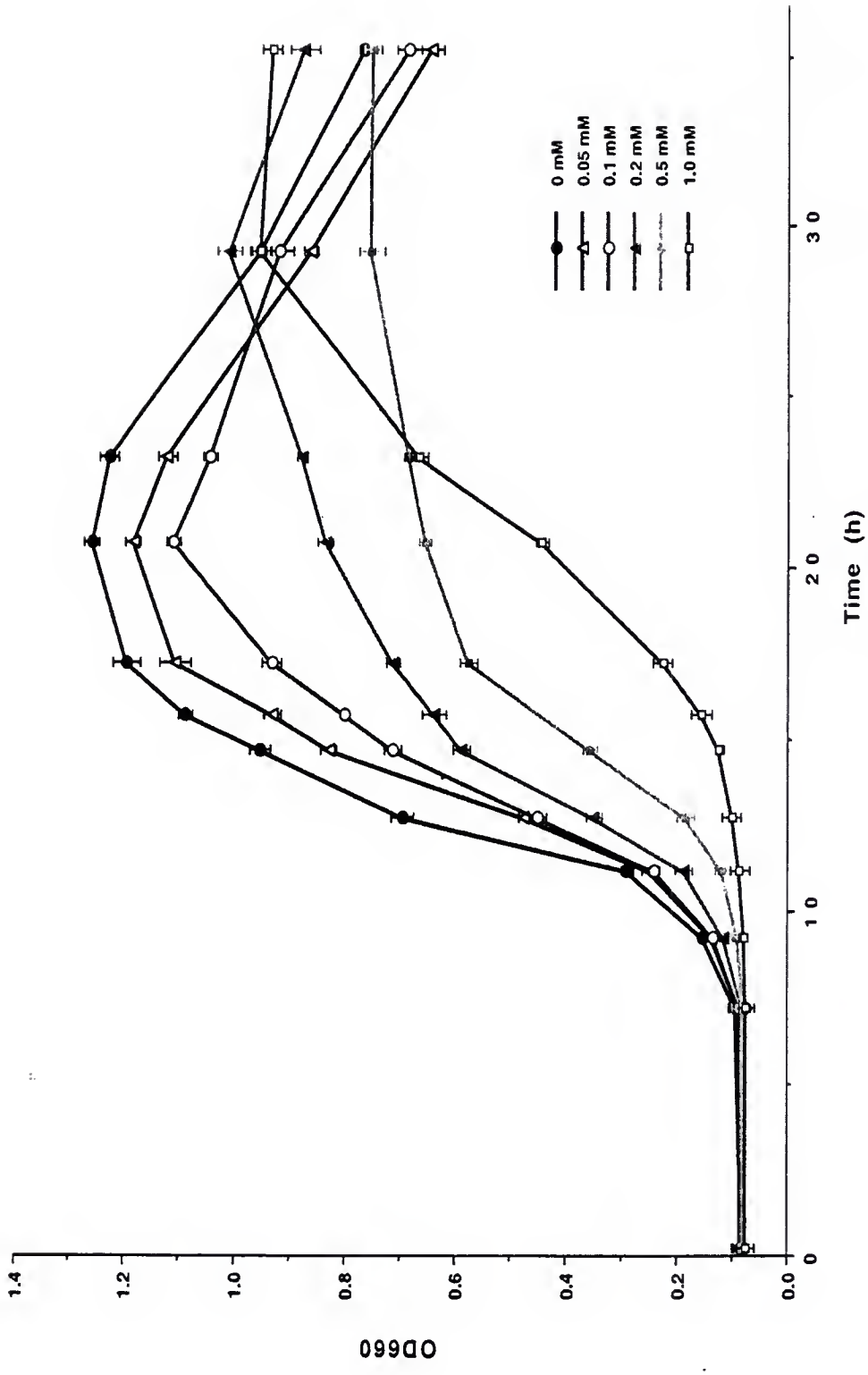


Figure 5-2. Effect of 7-NH_2-SPD on *P. aeruginosa* growth. Bacteria were grown in the presence of 7-NH_2-SPD at various concentrations from 0.05 to 1.0 mM. Data are average values from a single experiment performed in duplicate. Bars indicated ranges.

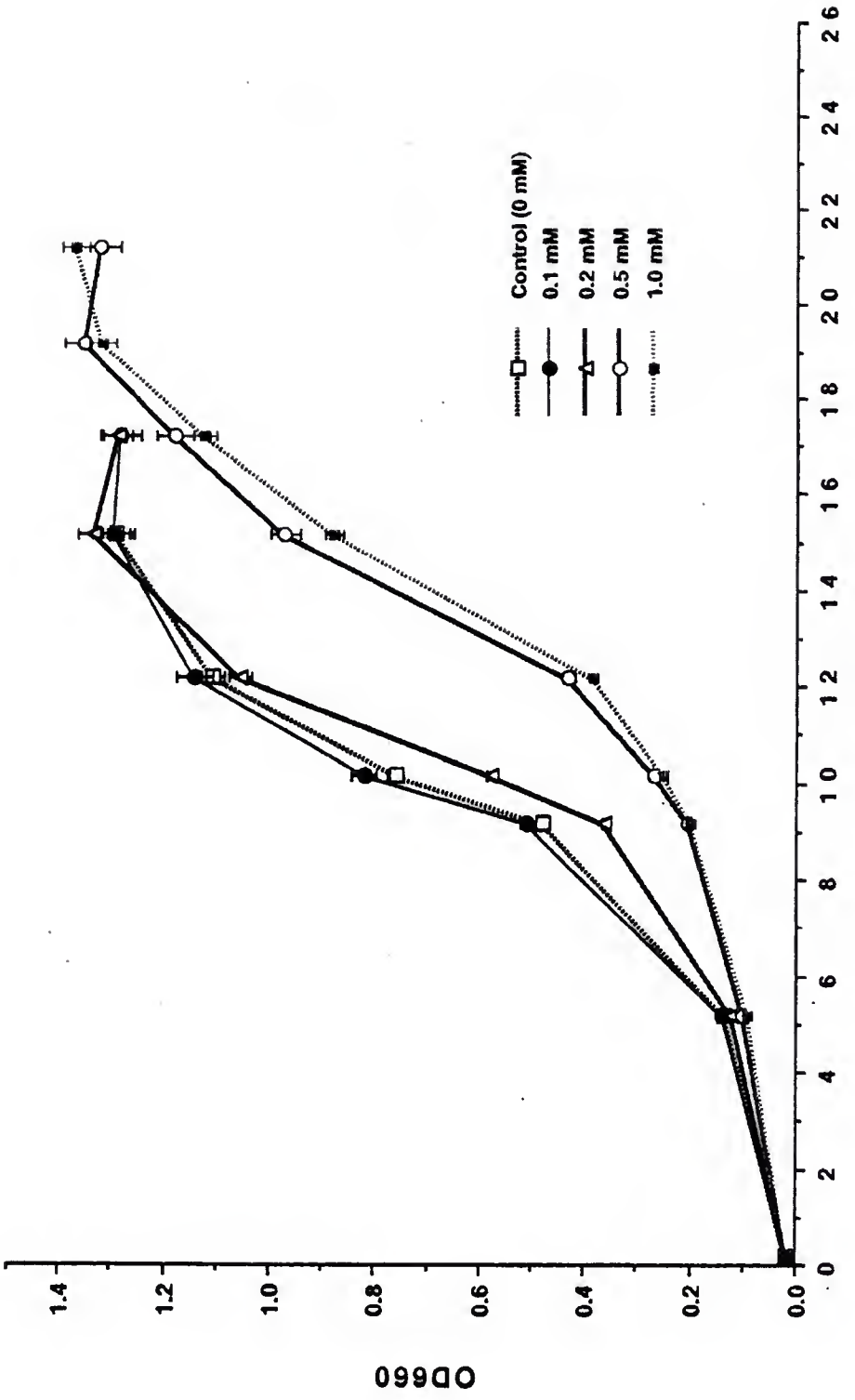


Figure 5-3. Effect of 7-NH_2-DESPD on *P. aeruginosa* growth. Bacteria were exposed to 7-NH_2-DESPD at various concentrations from 0.10 to 1.0 mM. Data are average values from a single experiment performed in duplicate. Bars indicated ranges.

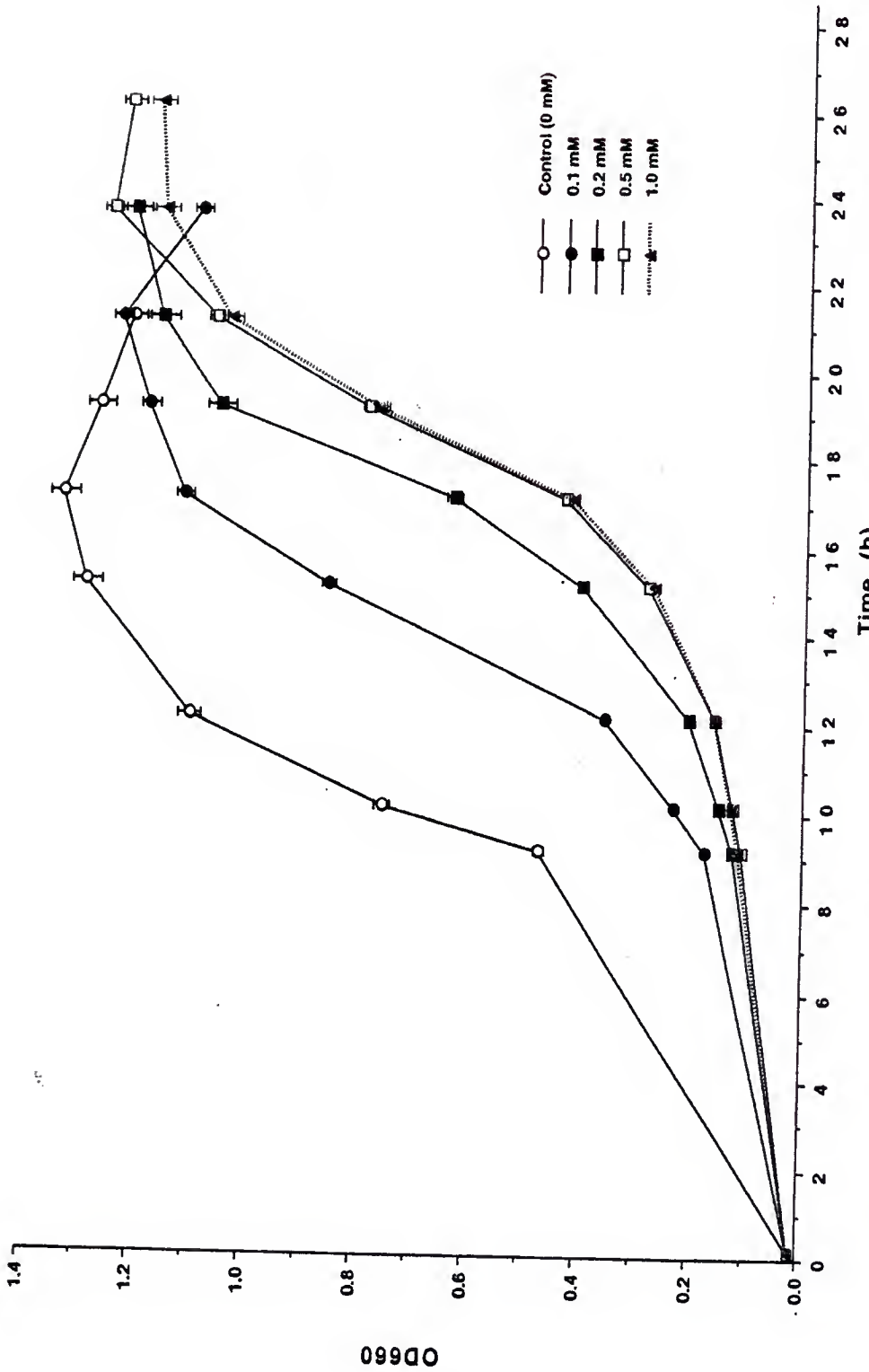


Figure 5-4. Effect of 6-NH_2-DESPM on *P. aeruginosa* growth. Bacteria were exposed to 6-NH_2-DESPM at various concentrations from 0.10 to 1.0 mM. Data are average values from a single experiment performed in duplicate. Bars indicated ranges.

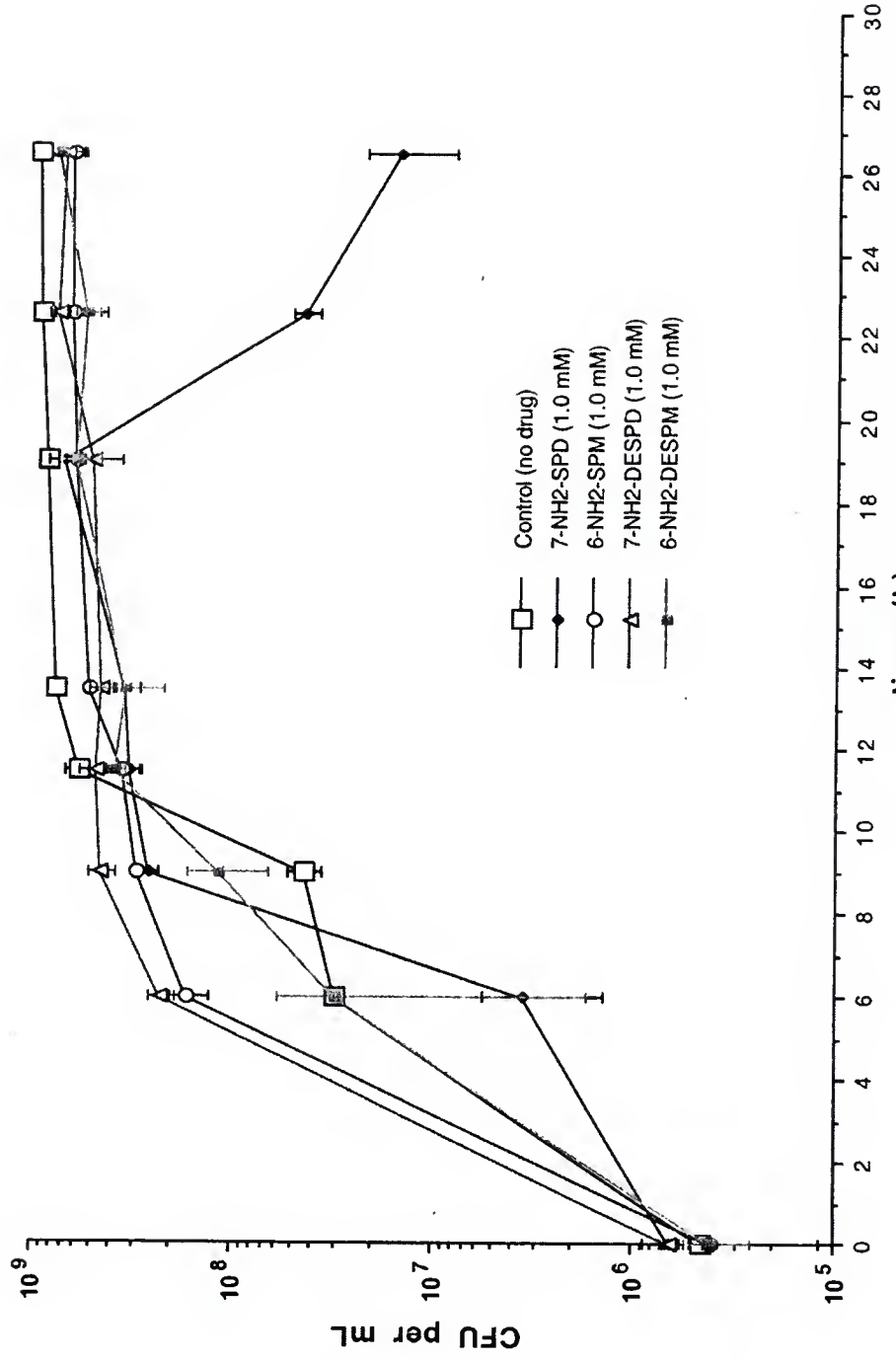


Figure 5-5. Effects of various aminopolyamines on *P. aeruginosa* colony forming units (CFU). Bacteria were grown in the presence of each indicated aminopolyamine at 1.0 mM. Error bars indicated standard deviations (n = 3).

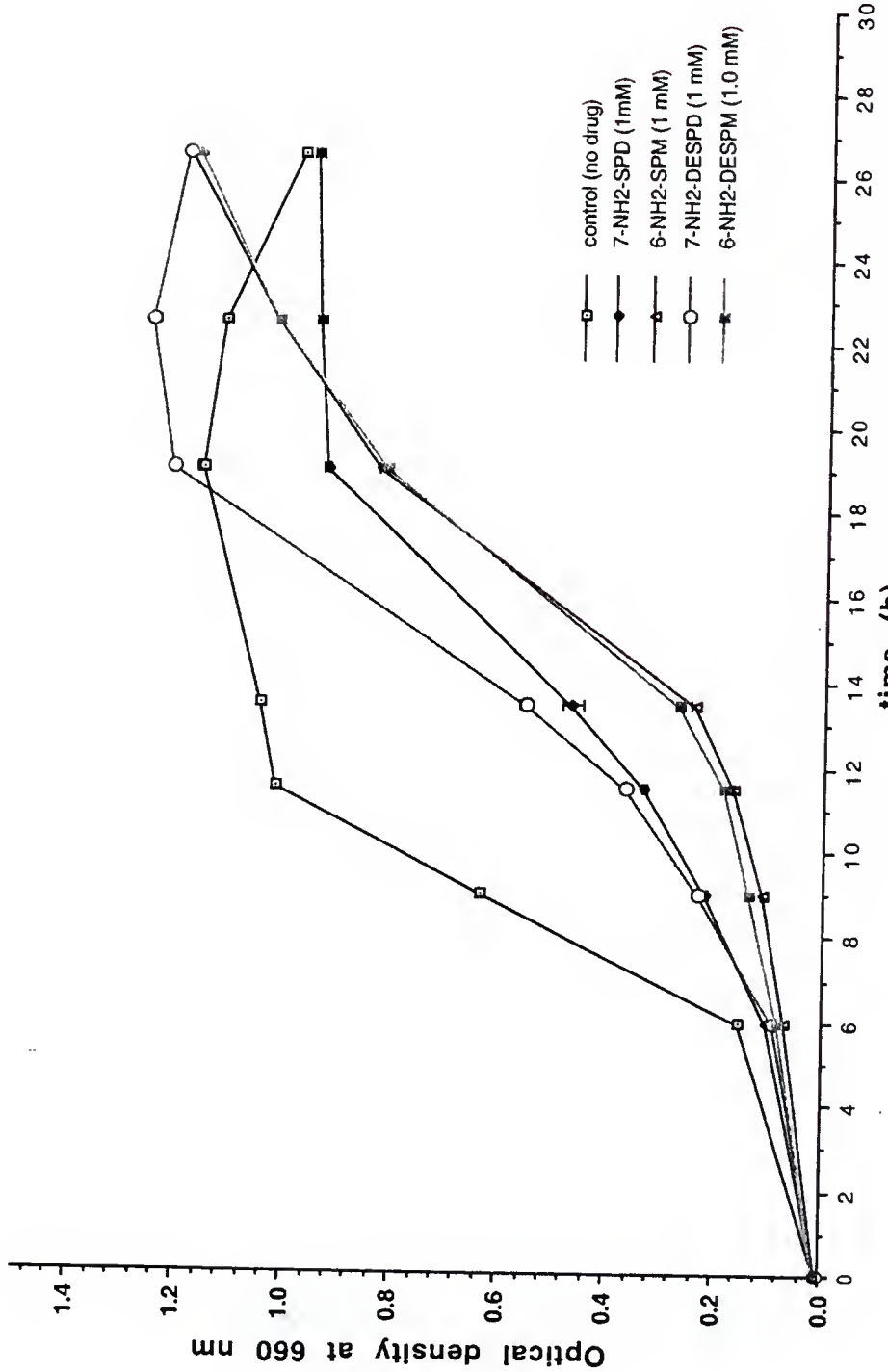


Figure 5-6. Effects of various aminopolyamines on *P. aeruginosa* growth. Bacteria were grown in the presence of each indicated aminopolyamine at 1.0 mM and the turbidity was described as optical density at 660 nm (OD_{660}). Error bar indicated standard deviations (n = 3).

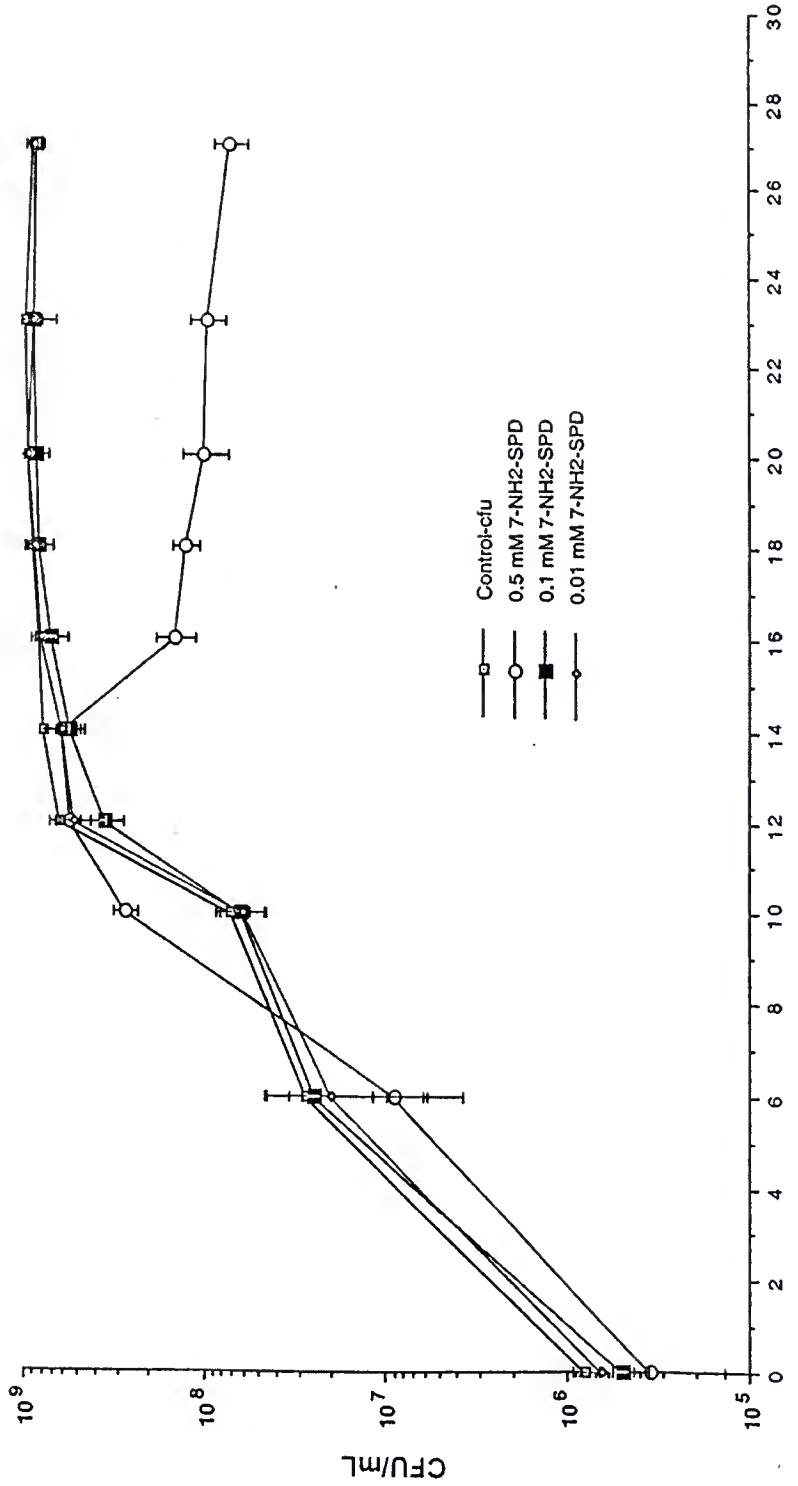


Figure 5-7. Effects of 7-NH_2-SPD on *P. aeruginosa* colony forming units (CFU). Bacteria were grown in the presence of 7-NH_2-SPD at three different concentrations (0.01 to 0.50 mM). Error bars indicated standard deviations (n = 3).

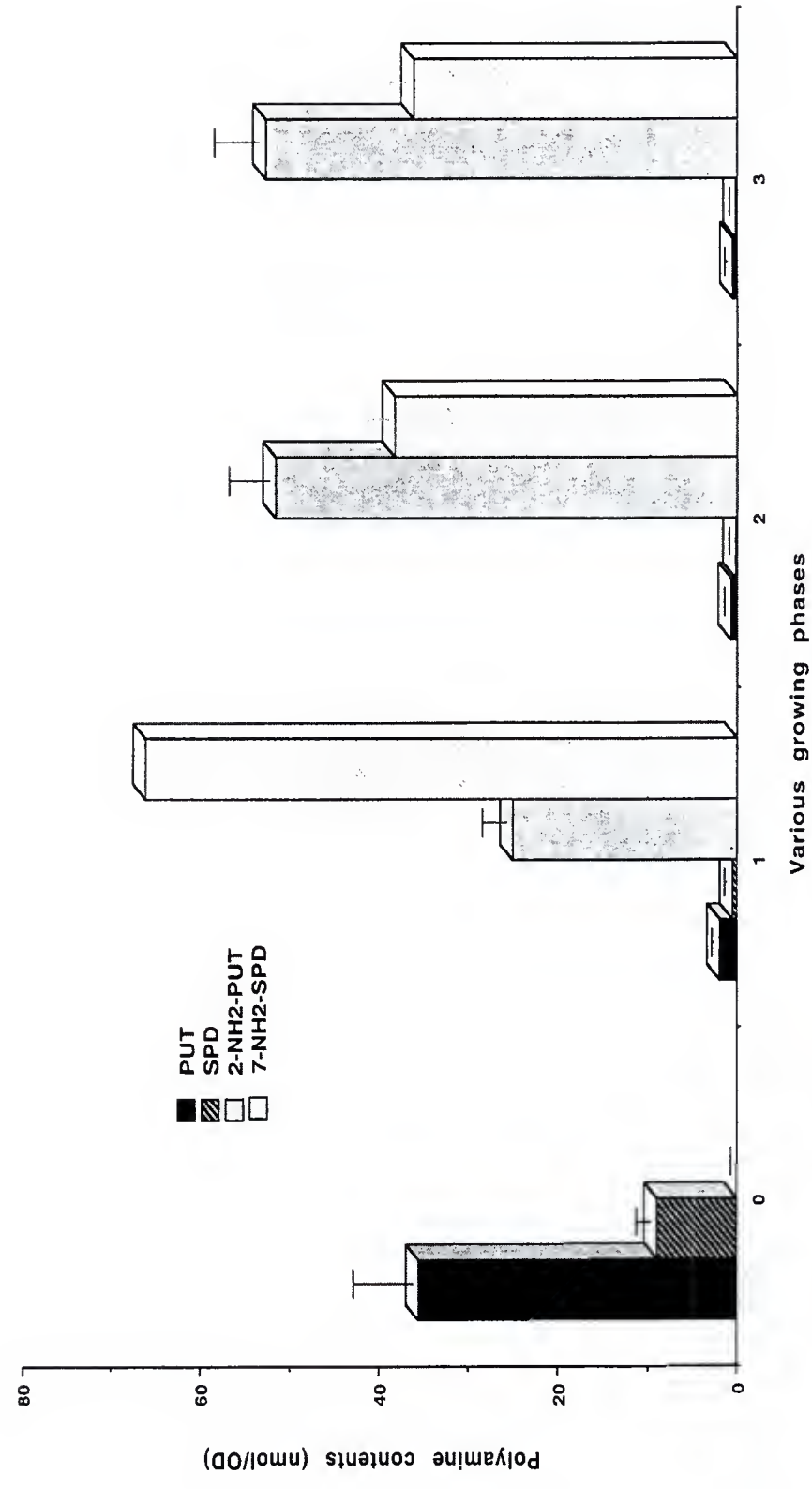


Figure 5-8. A comparison of polyamine levels in *P. aeruginosa* harvested from different growth phases. Bacteria were exposed to 7-NH_2-SPD (1.0 mM) and samples were collected at different phases for polyamine analyses. Horizontal axis indicates control, 0; early log phase, 1; mid log phase, 2; and stationary phase, 3. Data are results from a single experiment performed in duplicate. Error bars indicated ranges.

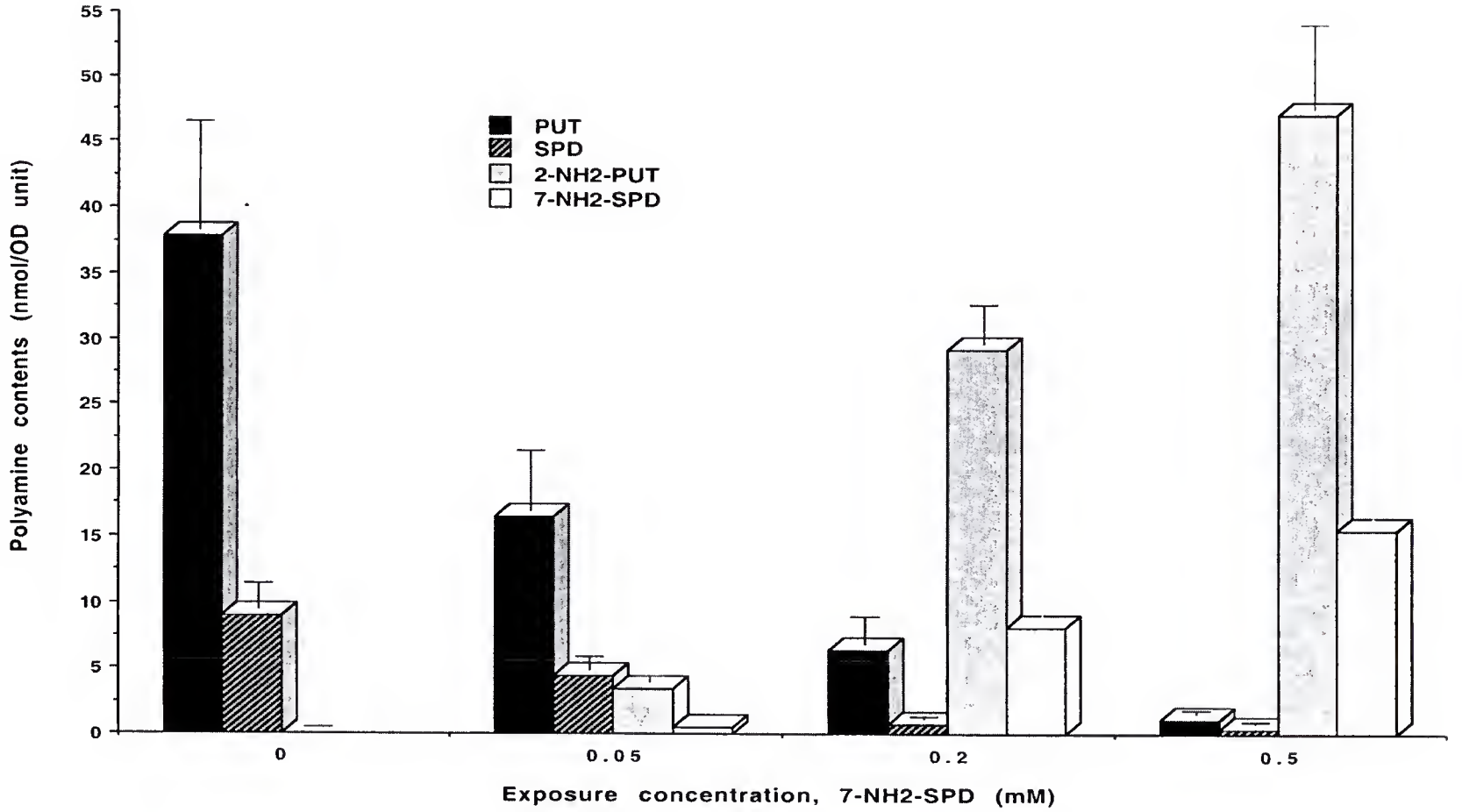


Figure 5-9. Effect of 7-NH_2-SPD on polyamine contents of *P. aeruginosa*. Bacteria were grown in the presence of 7-NH_2-SPD at three different concentrations (0.05 to 0.50 mM) and harvested at stationary phases for polyamine content analyses. Data are results from a single experiment performed in duplicate. Error bars indicated ranges.

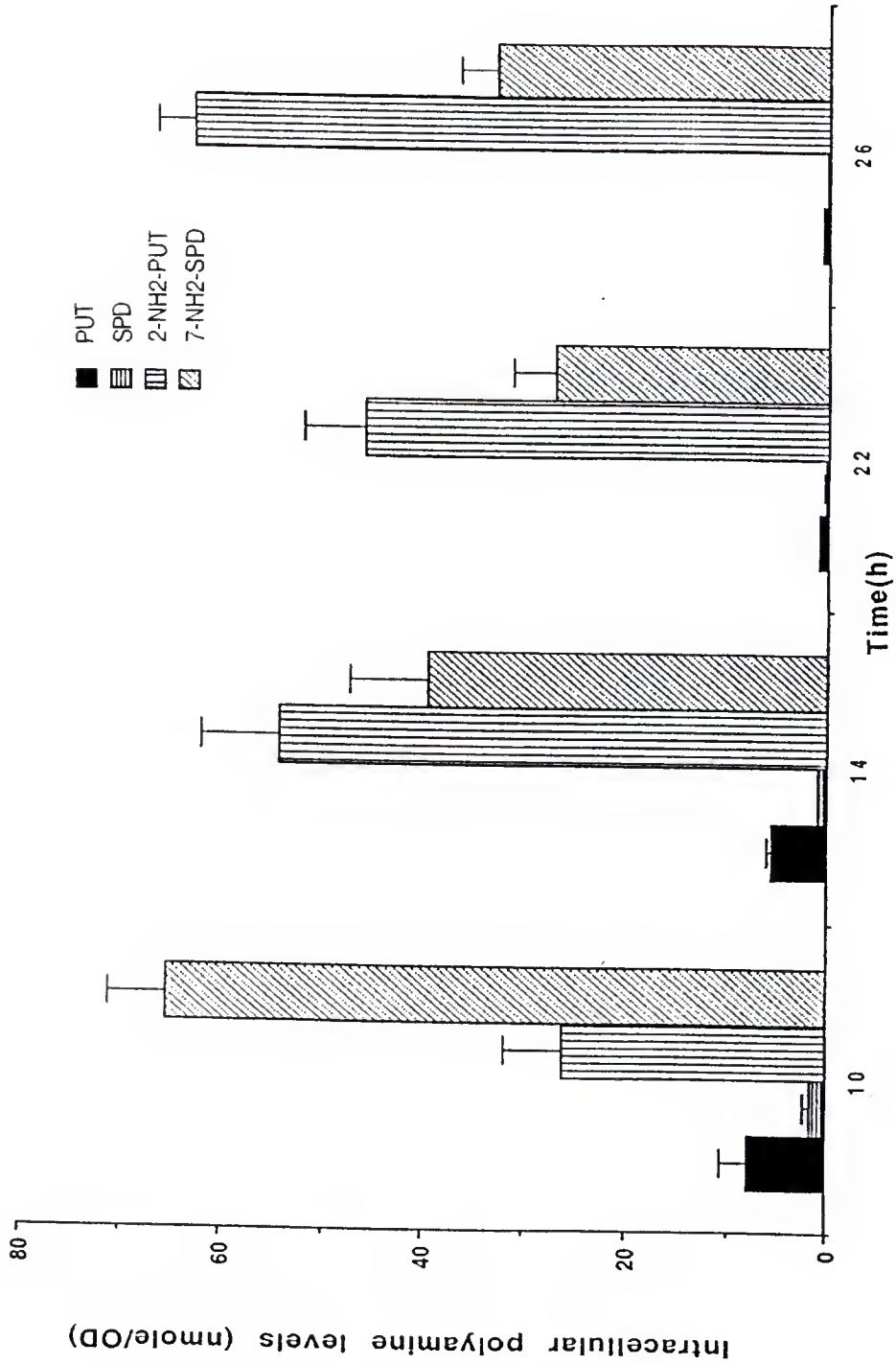


Figure 5-10. Polyamine levels in *P. aeruginosa* after different exposure duration. Bacteria were exposed to 7-NH_2-SPD (1.0 mM) and samples were collected at different time points for polyamine content analyses. Error bar indicated standard deviations (n = 3).

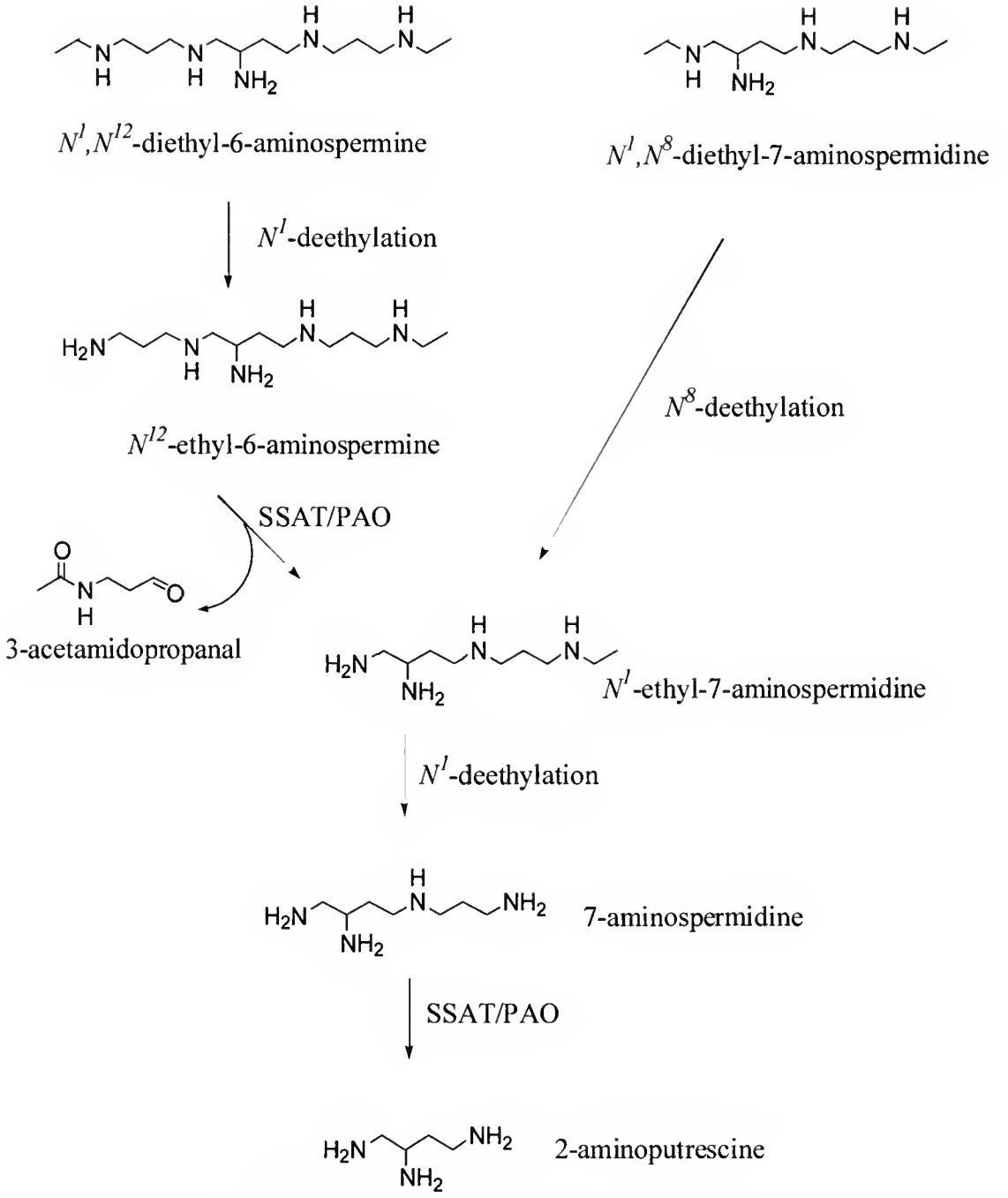


Figure 5-11. Metabolic breakdown of N^1,N^{12}-diethyl-6-aminospermine and N^1,N^8-diethyl-7-aminospermidine in *P. aeruginosa*.

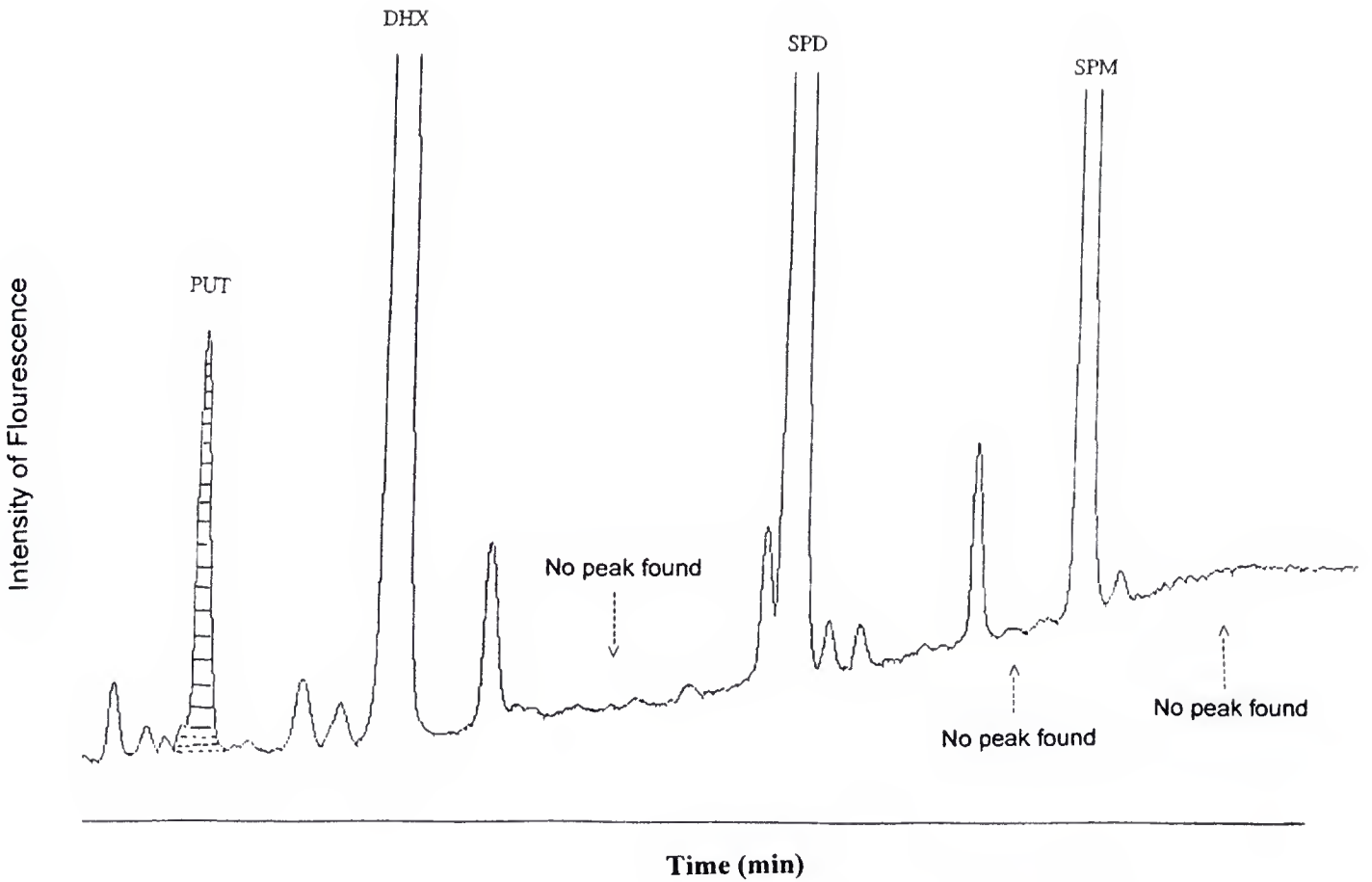


Figure 5-12. HPLC chromatograph of polyamine contents of L1210 cells (control, no drug). L1210 cells were grown in complete culture medium (without 7-NH_2-SPD) for 48 h and then harvested for polyamine pool analysis.

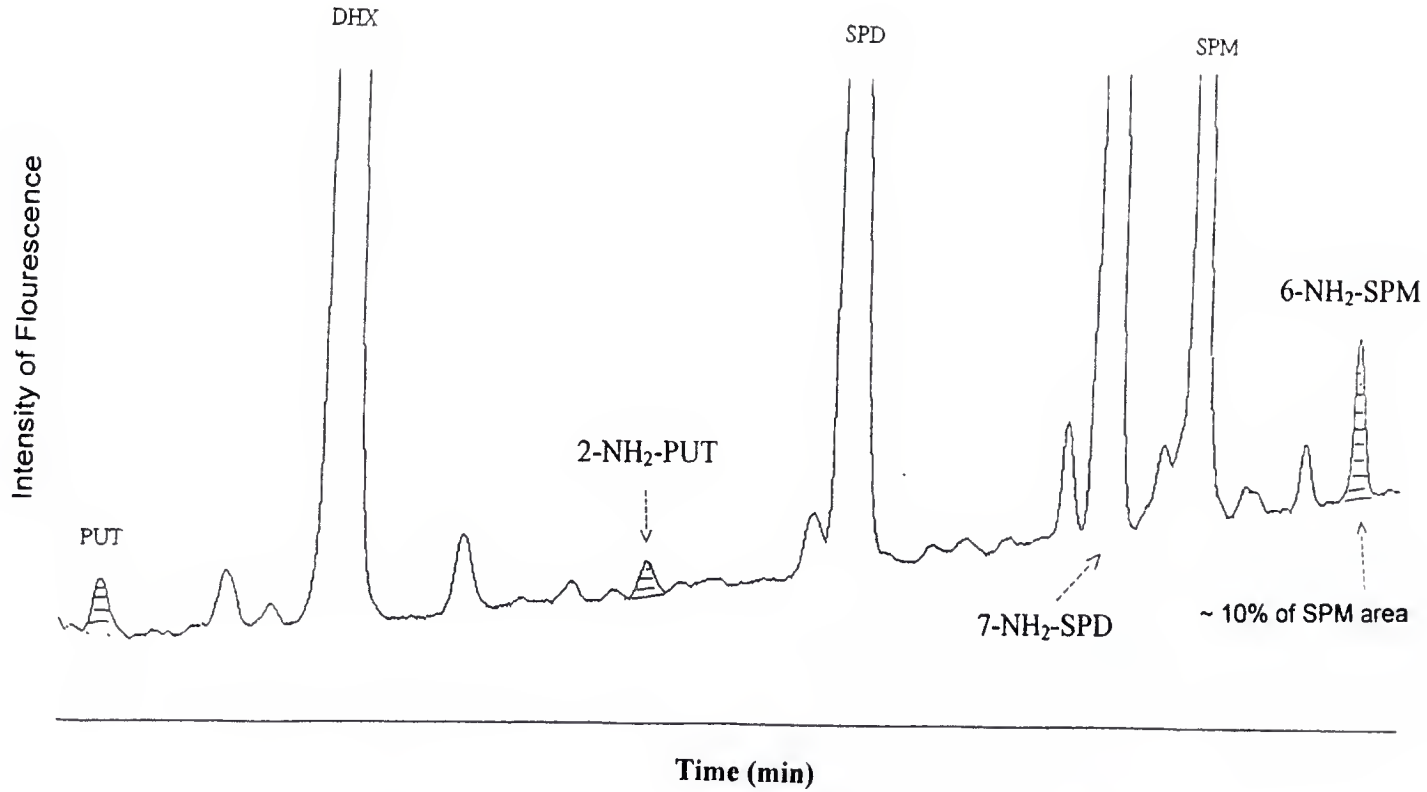


Figure 5-13. HPLC chromatograph of polyamine contents of L1210 cells exposed to 7-NH_2-SPD. Cells were grown in complete culture medium with 60 μM 7-NH_2-SPD for 48 h and then harvested for polyamine pool analysis. SPM was identified at a retention time of 27.1 min with an area of 92000 while 6-NH_2-SPM was identified at 29.9 min with an area of 9176 (~ 10% of SPM area).

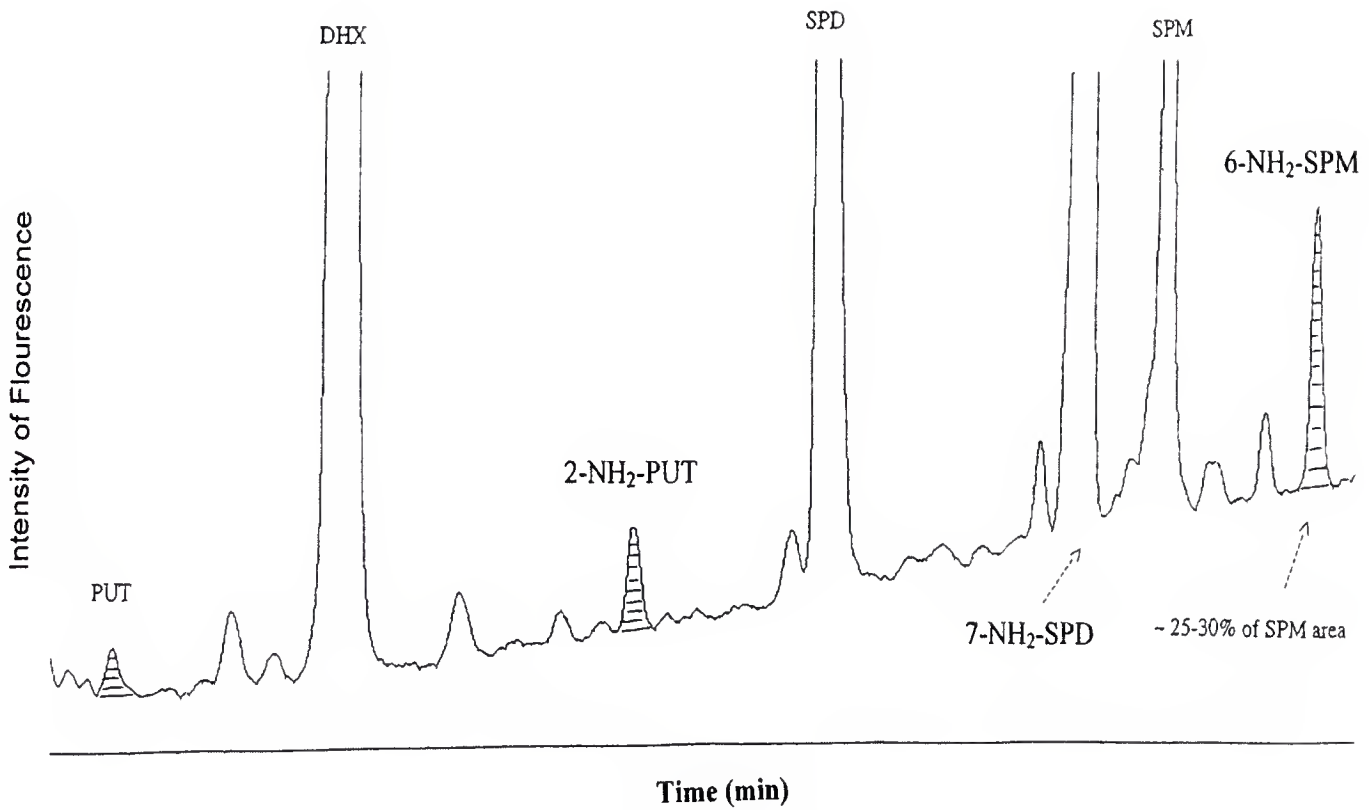


Figure 5-14. HPLC chromatograph of polyamine contents of L1210 cells exposed to 7-NH_2-SPD. Cells were grown in complete culture medium with 300 μM 7-NH_2-SPD for 48 h and then harvested for polyamine pool analysis. SPM was identified at a retention time of 27.2 min with an area of 75000 while 6-NH_2-SPM was identified at 29.9 min with an area of about 20000 (~ 25%-30% of SPM area).

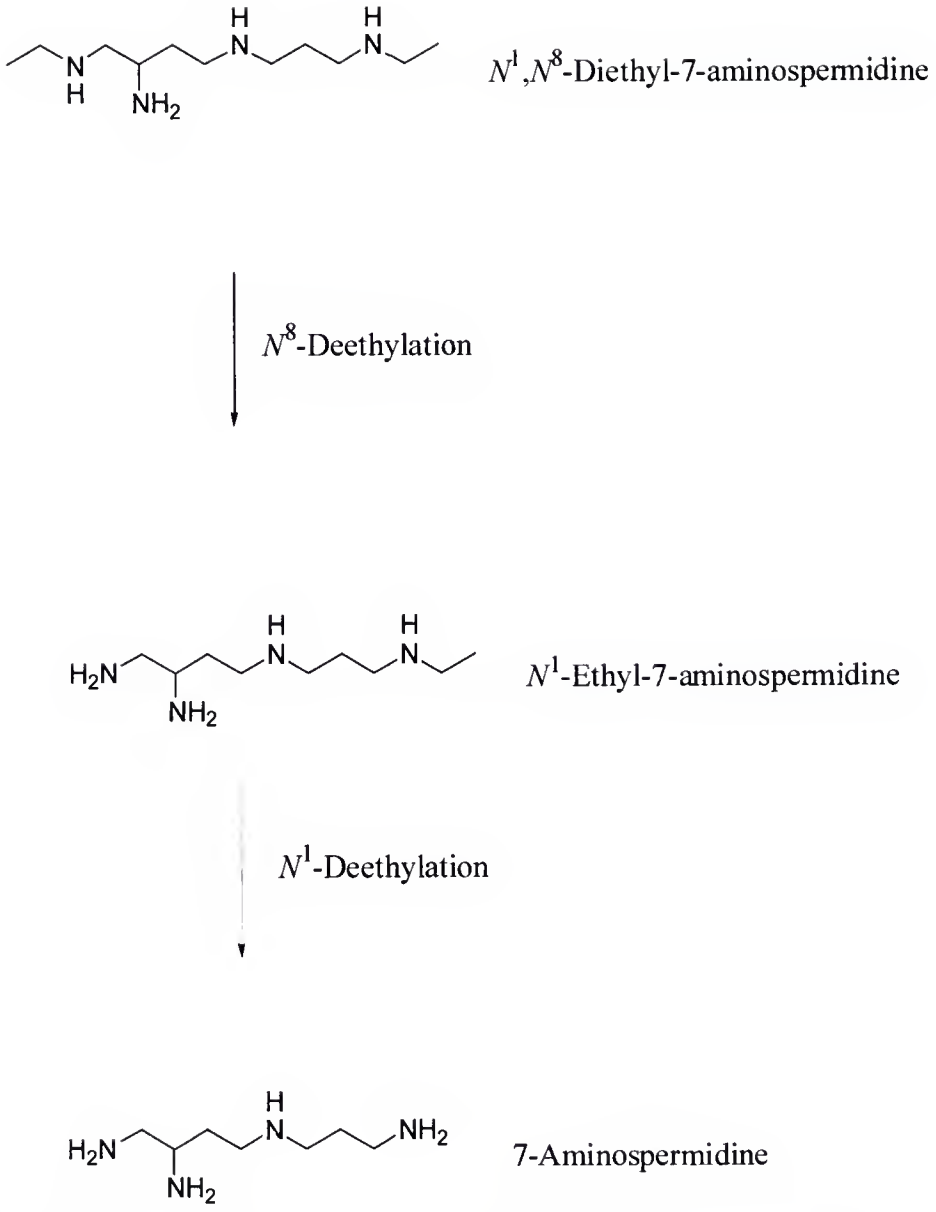


Figure 5-15. Metabolic breakdown of N^1,N^8-diethyl-7-aminospermidine in murine leukemia L1210 cells.

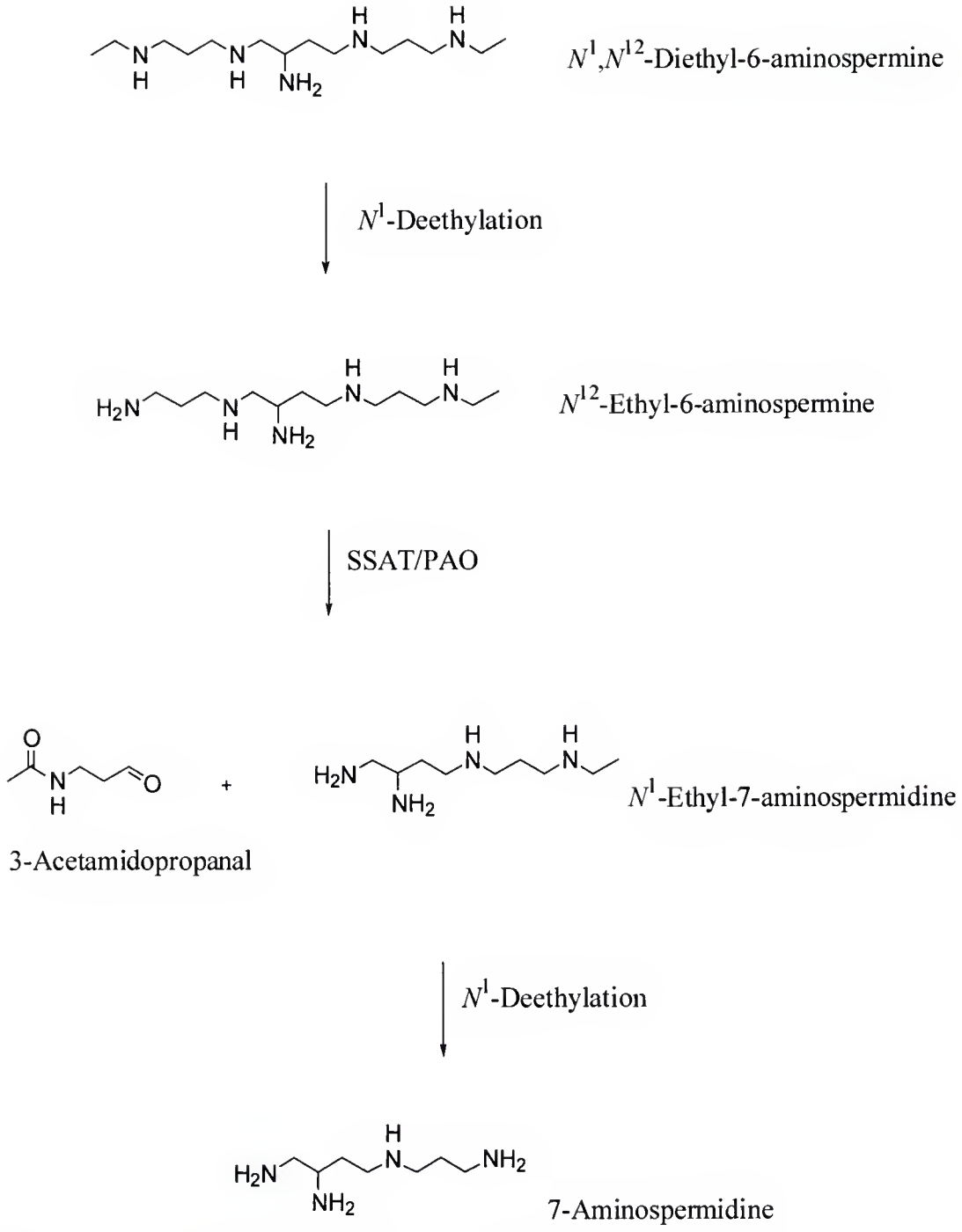


Figure 5-16. Metabolic breakdown of N^1,N^{12}-diethyl-6-aminospermine in murine leukemia L1210 cells.

Table 5-1. Total intracellular amine equivalence in *P. aeruginosa* after exposure to aminopolyamines. [a]Total nitrogen charge equivalents are calculated based on the assumption that 2-NH_2-PUT is +3, 7-NH_2-SPD or 7-NH_2-DESPD is +4, and 6-NH_2-SPM or 6-NH_2-DESPM is +5. [b]Total nitrogen charge equivalents are calculated based on the assumption that 2-NH_2-PUT is +2, 7-NH_2-SPD or 7-NH_2-DESPD is +3, and 6-NH_2-SPM or 6-NH_2-DESPM is +4 (in this case the charge of each aminopolyamine is the same as its corresponding natural polyamine parent, i.e., PUT, SPD, and SPM are +2, +3, and +4, respectively).

Aminopolyamine (exposure concentration, 1.0 mM)	Total charges (nanoequivalents/OD unit)[a]	Total charges (nanoequivalents/OD unit)[b]
Control (no drug)	112.24 ± 15.36	112.24 ± 15.36
2-NH_2-PUT	242.18 ± 11.34	158.93 ± 6.15
7-NH_2-SPD	272.87 ± 51.94	169.94 ± 30.52
7-NH_2-DESPD	209.42 ± 11.48	172.72 ± 8.15
6-NH_2-SPM	209.39 ± 8.78	164.04 ± 4.95
6-NH_2-DESPM	239.70 ± 26.73	180.01 ± 20.50

Table 5-2. L1210 growth inhibition and transport of various aminopolyamines. Each IC_{50} was estimated from the growth curve of L1210 cells grown in the presence of 9 different concentrations of each aminopolyamine: 0, 0.03, 0.1, 0.3, 1.0, 3, 10, 30, and 100 μM. K_i was determined based on simple substrate-competitive inhibition of radiolabeled SPD transport by L1210 cells.

Aminopolyamines	48-h IC_{50} (μM)	96-h IC_{50} (μM)	K_i (μM)
2-NH_2-PUT	> 100	> 100	> 500
7-NH_2-SPD	60	9	84
6-NH_2-DESPM	9	0.35	33.7
6-NH_2-SPM	> 100	10	21
7-NH_2-DESPD	100	6	130

Table 5-3. Impact of aminopolyamines on polyamine metabolic enzymes of L1210 cells. All values are given in percent of control enzyme activity in which cells were untreated. Enzyme assays are described in detail in chapter 6. Data are results from experiments performed in triplicate and repeated at least once ($n \geq 6$).

Aminopolyamines	ODC	AdoMetDC	SSAT
7-NH_2-SPD	72% $\pm$ 4%	81% $\pm$ 2%	189% $\pm$ 8%
6-NH_2-SPM	52% $\pm$ 2%	70% $\pm$ 3%	117% $\pm$ 6%
7-NH_2-DESPD	79% $\pm$ 3%	86% $\pm$ 4%	303% $\pm$ 12%
6-NH_2-DESPM	19% $\pm$ 2%	53% $\pm$ 4%	668% $\pm$ 16%

Table 5-4. Impact of 7-aminospermidine on polyamine pools of L1210 cells. Putrescine (PUT), spermidine (SPD), and spermine (SPM) levels after 48 h of exposure are reported. The concentration unit is pmol/10^6 cells. The numbers in parentheses indicate the percentage of control (untreated cells). Intracellular concentration of 7-aminospermidine (7-NH_2-SPD) and its metabolite 2-aminoputrescine (2-NH_2-PUT) are also reported. Each experiment was performed in triplicate.

Treatment	PUT	SPD	SPM	2-NH_2-PUT	7-NH_2-SPD
Control	146 ± 21	3046 ± 223	837 ± 74	0.00	0.00
60 μM 7-NH_2-SPD	51 ± 15 (35 ± 10)	1022 ± 108 (34 ± 4)	644 ± 72 (77 ± 9)	35 ± 24	2537 ± 349
300 μM 7-NH_2-SPD	35 ± 7 (24 ± 5)	557 ± 91 (18 ± 3)	525 ± 97 (63 ± 12)	29 ± 21	3630 ± 898

Table 5-5. Impact of N^1,N^8-diethyl-7-aminospermidine on polyamine pools of L1210 cells. Putrescine (PUT), spermidine (SPD), and spermine (SPM) levels after 48 h of exposure are reported. The concentration unit is pmol/10^6 cells. The numbers in parentheses indicate the percentage of control (untreated cells). Intracellular levels of N^1,N^8-diethyl-7-aminospermidine (7-NH_2-DESPD) and its metabolites N^1-ethyl-7-aminospermidine (7-NH_2-MESPD), 2-aminoputrescine (2-NH_2-PUT), and 7-aminospermidine (7-NH_2-SPD) are also reported. Each experiment was performed in triplicate.

Treatment	PUT	SPD	SPM	7-NH_2-DESPD	7-NH_2-MESPD	2-NH_2-PUT	7-NH_2-SPD
Control	115 ± 15	2591 ± 262	752 ± 71	0	0	0	0
100 μM 7-NH_2-DESPD	64 ± 2 (56 ± 1)	751 ± 2 (29 ± 0)	454 ± 7 (60 ± 1)	1765 ± 94	155 ± 7	0	7 ± 3
500 μM 7-NH_2-DESPD	50 ± 4 (43 ± 4)	457 ± 29 (18 ± 1)	366 ± 29 (49 ± 4)	2702 ± 178	214 ± 39	0	12 ± 5

Table 5-6. Impact of N^1,N^{12}-diethyl-6-aminospermine on polyamine pools of L1210 cells. Putrescine (PUT)), spermidine (SPD), and spermine (SPM) levels after 48 h of exposure are reported. The concentration unit is pmol/10^6 cells. The numbers in parentheses indicate the percentage of control (untreated cells). Intracellular levels of N^1,N^{12}-diethyl-6-aminospermine (6-NH_2-DESPM) and its metabolites N^1-ethyl-7-aminospermidine (7-NH_2-MESPD), 2-aminoputrescine (2-NH_2-PUT), and 7-aminospermidine (7-NH_2-SPD) are also reported. Each experiment was performed in triplicate (Bergeron, R. J.; Huang, G.; Yao, H., unpublished).

	PUT	SPD	SPM	6-NH_2-DESPM	7-NH_2-MESPD	2-NH_2-PUT	7-NH_2-SPD
Control	135 ± 19	2784 ± 254	798 ± 78	0	0	0	0
10 μM 6-NH_2-DESPM	57 ± 13 (39 ± 9)	480 ± 91 (16 ± 3)	451 ± 89 (54 ± 11)	3519 ± 726	54 ± 12	0	21 ± 5
50 μM 6-NH_2-DESPM	38 ± 1 (26 ± 1)	198 ± 11 (7 ± 0)	266 ± 7 (32 ± 1)	3105 ± 118	43 ± 7	0	23 ± 5

Table 5-7. Total intracellular amine equivalence in L1210 cells after exposure to aminopolyamines. [a]Total nitrogen charge equivalents are calculated based on the assumption that 2-NH_2-PUT is +3, 7-NH_2-SPD or 7-NH_2-DESPD is +4, and 6-NH_2-SPM or 6-NH_2-DESPM is +5. [b]Total nitrogen charge equivalents are calculated based on the assumption that 2-NH_2-PUT is +2, 7-NH_2-SPD or 7-NH_2-DESPD is +3, and 6-NH_2-SPM or 6-NH_2-DESPM is +4 (in this case the charge of each aminopolyamine is the same as its corresponding natural polyamine parent, i.e., PUT, SPD, and SPM are +2, +3, and +4, respectively).

Aminopolyamine (exposure concentration)	Total picoequivalents/10^6 cells (x 10^3)[a]	Total picoequivalents/10^6 cells (x 10^3)[b]
Control (no drug)	12.78 ± 1.01	12.78 ± 1.01
7-NH_2-SPD (60 μM)	16.00 ± 2.11	13.43 ± 1.74
7-NH_2-SPD (300 μM)	18.45 ± 4.33	14.79 ± 3.41
7-NH_2-DESPD (100 μM)	11.91 ± 0.46	9.98 ± 0.35
7-NH_2-DESPD (500 μM)	14.65 ± 1.10	11.72 ± 0.88
6-NH_2-DESPM (10 μM)	21.25 ± 4.35	17.66 ± 3.61
6-NH_2-DESPM (50 μM)	17.52 ± 0.70	14.35 ± 0.56

CHAPTER 6
EXPERIMENTAL METHODS: BIOLOGICAL STUDIES, ANALYTICAL DEVELOPMENTS AND SYNTHESES OF AMINOPOLYAMINES

This chapter describes materials and methods of all cellular studies including cell growths, enzyme activities, uptakes and polyamine pools. Bacterial studies including bacterial growth, CFU (colony forming units), and polyamine content analyses are described. The detailed procedure of analytical methods and syntheses of aminopolyamines are also discussed in this chapter.

Cellular Studies

The murine leukemia cell line L1210 was purchased from American Type Culture Collection (ATCC) (CCL 219). RPMI-1640 medium, fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 3-(N-morpholino)-propanesulfonic acid (MOPS) were obtained from Gibco (Grand Island, NY). Cells were maintained in logarithmic growth in complete culture medium containing RPMI-1640, fetal bovine serum, HEPES, and MOPS at 37 ^{0}C in a water-jacketed 5% CO_2 incubator. Cell number was determined by electronic particle counting (model Z_F coulter counter, Coulter Electronics, Hialeah, FL).

IC_{50} Determinations

L1210 cells were grown in 25-mL culture flasks. Cultures were treated while in logarithmic growth (0.5-1.0 x 10^5 cells/mL) with the aminopolyamines dissolved in sterile water and filtered through a 0.2-μm syringe filter. After a 48-h period, cell

numbers were counted to determine 48-h IC_{50} values and in the case of 96-h IC_{50} determination, cells were reseeded and incubated for an additional 48 h. The percentage of control growth was determined as follows:

$$\% \text{ of control growth} = \frac{\text{final treated cell number - initial inoculum}}{\text{final untreated cell number - initial inoculum}} \times 100$$

The IC_{50} is the concentration of a compound at which cell growth is reduced to 50% of control growth after defined intervals of exposure (e.g., 48 h and 96 h). Each IC_{50} value was estimated at the intersecting point of IC_{50} curve and the 50% line (Fig. 6-9, Fig. 6-10, Fig. 6-11, and Fig. 6-12).

Polyamine Pool Analysis

Cells in logarithmic growth were exposed to each aminopolyamine compound at IC_{50} concentration and 5 times of that concentration for 48 h. At the end of the exposure period, cell suspensions were sampled, washed twice in ice-cold buffered water and centrifuged to obtain pellets. The pellets were subjected to polyamine extraction using 0.6 *N* perchloric acid (1 mL/10^7 cells) followed by freeze-fracturing in liquid nitrogen/hot water three times (Bergeron et al., 1994). After centrifugation to remove cellular debris, the supernatant was collected for HPLC analysis of polyamine content by the dansylation method described in detail in another section of this chapter.

Uptake Determinations

Aminopolyamines were studied for their ability to compete with radiolabeled SPD for uptakes into the leukemia L1210 cells. L1210 cells were incubated in 1 mL of culture medium containing 1, 2, 4, 6, 8, and 10 μM radiolabeled SPD alone (the control experiment) or in the presence of 10, 25, and 50 μM aminopolyamine for 20 min at 37 ^{0}C in a water bath with gentle shaking. The tubes were then centrifuged at 1000 rpm for 5

min at 0-4 ^{0}C. The pellet was washed twice with cold RPMI-1640 containing 1 mM SPD, digested with 200 μL of 0.1% Triton X-100 and vortexed well. The entire content was transferred to a vial for scintillation counting. From Lineweaver-Burke plots K_i, representing competitive inhibition with respect to SPD, was calculated according to the following formula:

$$K_i = \frac{\text{Concentration of studied compound}}{\frac{K_{apparent}}{K_m} - 1}$$

Ki plots of various aminopolyamines are shown in Fig. 6-13, Fig 6-14, Fig. 6-15, and Fig. 6-16.

Enzyme Assays

ODC and AdoMetDC activities were routinely determined by measuring the $^{14}CO_2$ released from [^{14}C]carboxyl-labeled L-ornithine or S-adenosyl-L-methionine (Porter, 1987 and 1990).

ODC assay

Buffer A. Buffer A consists of TrisCl (25 mM), EDTA (100 μM), dithiothreitol (DTT) (1.25 mM), pyridoxal phosphate (125 μM). NaOH (1 N) was used to solubilize EDTA and adjust pH to 7.2, at which buffer A was a bright yellow solution.

Buffer B. Buffer B contains L-ornithine in buffer A (387 μg/mL)

Stock substrate. Substrate solution contains 34.8 μL of [^{14}C]L-ornithine in 965.2 μL of buffer B. This stock substrate solution contains 2.25 mM of the radiolabeled L-ornithine.

ODC preparation. L1210 cells were grown in 200 mL of complete culture medium with the addition of an aminopolyamine at a concentration of 1 μM. N^1,N^{14}-Diethylhomospermine (DEHSPM), which is known to reduce ODC activity to 5-10% of control, served as a positive control (Seely, 1983) (from this point on, negative control is also known as control, which is the untreated cell sample). After 4 h of treatment, L1210 cells were centrifuged at 1200 rpm and 0 ^{0}C for 10 min. The medium was discarded properly and the pellets were resuspended in 40 mL of incomplete medium. Each cell suspension was centrifuged again, and the medium was discarded. The pellets were resuspended in 30.4 mL of incomplete medium. Four hundred microliters of the cell suspension was used to determine the cell counts. The remaining 30 mL of the cell suspension was centrifuged, and the medium was removed completely without disturbing the cell pellets. The pellets were resuspended in buffer A solution (4×10^7 cells/mL) and homogenized by high energy ultrasonication for 15 seconds. The whole content was centrifuged at 10000 g (speed 7-8) and 4 0 °C for 5 min. The supernatant (800 μL) was transferred to a small tube and was used for the ODC assay as described below

ODC assay procedure. A 200 μL portion of the supernatant containing ODC was added to a 10-mL assay flask equipped with a plastic well containing Whatman 3 mm filter paper and 0.2 mL of benzethonium hydroxide. Stock substrate solution (50 μL) was then added. The flask was capped securely with two rubber septa and incubated at 37 °C for 30 min with shaking at 10 cycles per min. After cooling in an ice bath for 5 min, 0.3 mL of 1 M H_2SO_4 was added to the assay mixture to stop the reaction and to liberate $^{14}CO_2$ from the solution. The resulting assay solution was incubated at 37 °C for 15 min to complete the absorption of $^{14}CO_2$ to the filter paper. The whole plastic well

with filter paper was placed into a scintillation vial containing 15 mL scintillation fluid. The vial was mixed well and counted for radioactivity on the next day.

AdoMetDC assay

Buffer B1. Buffer B1 contains 18.4 mg of PUT in 5.636 mL of above buffer A

Stock substrate. A 296 μL of commercially available 0.39 mM [^{14}C]adenosylmethionine was added to a solution of buffer B1 (879 μL) containing adenosylmethionine (1.54 mg) and 1 *N* NaOH (9.48 μL). The resulting 1.184 mL of stock substrate was used for the AdoMetDC assay.

AdoMetDC preparation. L1210 cells were grown in 200 mL of complete culture medium with the addition of an aminopolyamine at a concentration of 1 μM. Diethylhomospermine (DEHSPM), which is known to reduce AdoMetDC activity to 50% of control, served as a positive control. After six hours of treatment, L1210 cells were centrifuged at 1200 rpm and 0 ^{0}C for 10 min. The medium was discarded properly, and the pellets were resuspended in 40 mL of incomplete media. The cell suspension was centrifuged again and the medium was discarded. The pellets were resuspended in 30.4 mL of incomplete medium. Four hundred microliters of the cell suspension was used to determine the cell counts. The remaining 30 mL of the cell suspension was centrifuged, and the medium was removed completely without disturbing the cell pellets. The pellets were resuspended in buffer A solution (4×10^7 cells/mL) and homogenized by high energy ultrasonication for 15 seconds. The whole content was centrifuged at 10000 g (speed 7-8) and 4 0 ^{0}C for 5 min. The supernatant (800 μL) was transferred to a small tube and was used for the AdoMetDC assay as described below.

AdoMetDC assay procedure. A 200 μL-portion of the supernatant containing AdoMetDC was added to a 10-mL assay flask equipped with a plastic well containing Whatman 3 mm filter paper and 0.2 mL of benzethonium hydroxide. Stock substrate solution (50 μL) was then added. The flask was capped securely with two rubber septa and incubated at 37 °C for 30 min with shaking at 10 cycles per min. After cooling in an ice bath for 5 min, 0.5 mL of 1 M H_2SO_4 was added to the assay mixture to stop the reaction and to liberate $^{14}CO_2$ from the solution. The resulting assay solution was incubated at 37 °C for 30 min to complete the absorption of $^{14}CO_2$ to the filter paper. The whole plastic well with filter paper was placed into a scintillation vial containing 15 mL scintillation fluid. The vial was mixed well and counted for radioactivity on the next day.

SSAT assay

Spermidine/Spermine N^1-acetyltransferase activity was evaluated based on the measurement of [^{14}C]-N^1-acetylspermidine formed by the acetylation of SPD with [^{14}C]acetyl coenzyme A according to a known method (Libby, 1989). DENSPM at a concentration of 2 μM, which is known to upregulate SSAT to 1200% of negative control (no drug), serves as a positive control.

Cellulose phosphate paper preparation. Cellulose phosphate paper was treated with 1 *N* HCl for 10-15 min, rinsed with deionized water until neutral, and air-dried.

Substrate solution. To a solution of 750 μL of 6 mM SPD trihydrochloride in 150 mM HEPES, 25 μL of ^{14}C-AcetylCoA was added. The resulting mixture was used as a substrate solution for SSAT enzymatic studies.

Enzyme preparation. L1210 cells were grown in 50 mL of complete medium with the presence of an aminopolyamine at a concentration of 10 μM. In the case of

positive control, cells were exposed to N^1,N^{11}-diethylnorspermine (DENSPM) at a concentration of 2 μM. After 48 h, the cell suspension was centrifuged and the pellets were washed twice with cold DPBS (an isotonic buffer solution, 2 x 20 mL). The pellets were then treated with homogenation buffer solution (400 μL/10^7 cells) (the homogenation buffer contains 5.0 mM HEPES and 1.0 mM DTT at pH 7.6). The suspension was homogenized by high energy ultrasonication for 15 seconds and centrifuged at 10000 g for 20 min. to remove cellular debris. The obtained supernatant was further centrifuged at 12000 g and 4 ^{0}C for 4 h. The resulting supernatant, also known as SSAT homogenate, was then used for the assay.

SSAT assay procedure. To a 0.4 mL microcentrifuge tube containing 50 μL of SSAT homogenate, 50 μL of substrate solution was added. The reaction mixture was incubated at 37 ^{0}C for 15 min. For a blank control, 50 μL of substrate solution was mixed with 50 μL of 150 mM HEPES solution. The enzymatic reaction was terminated by cooling to 0 ^{0}C, followed by treatment with 20 μL of 1.0 M $NH_2OH.HCl$. The resulting mixture was heated in a boiling water bath for 3 min, cooled to room temperature and centrifuged at 4 ^{0}C and 12000 g for 10 min. The obtained supernatant (50 μL) was applied to cellulose phosphate paper followed by soaking of the paper in distilled water overnight. After rinsing with deionized water and air-drying, the cellulose phosphate paper was transferred into a scintillation vial containing 15 mL scintillation fluid and the radioactivity was counted for 2 min.

Microbial Studies

Pseudomonas aeruginosa was obtained from ATCC (catalog # 97). A Steris Amsco Renaissance Remanufactured 3021 Gravity Steam Sterilizer was used to

autoclave all bacterial culture solutions. Nutrient Broth and Nutrient Agar were purchased from DIFCO Laboratories. Nutrient broth was prepared by mixing 8 g of nutrient broth powder with 1.0 L of distilled water. The nutrient broth solution was then autoclaved for 45 min at 121 ^{0}C and 19 psi prior to use. Nutrient agar plates were prepared from a solution of 23 g of nutrient agar in distilled water (1.0 L). The nutrient agar solution was autoclaved for 45 min at 121 ^{0}C and 19 psi. Bacteria were grown in a New Brunswich Scientific Classic Series C24 Incubator shaker. Nutrient agar plates with bacteria were incubated in a Forma Scientific Model 3029 S/N 31272-586 CO_2 incubator.

***Pseudomonas aeruginosa* Growth**

A lyophilized culture of *P. aeruginosa* was rehydrated in sterile conditions with 2 mL nutrient broth. All bacterial samples were incubated at 30 ^{0}C in a rotary shaker described above at 250 rpm. After three to four passages, when the bacterial growth curve has become normal, the bacteria were ready for experiments. The bacteria were subjected to growth curve observation with or without drugs added to nutrient broth. Growth curves were plotted from the bacterial turbidity determined by optical density at 660 nm (OD_{660}) versus time.

Colony Forming Units (CFU)

Pseudomonas aeruginosa was grown in nutrient broth with and without the addition of one of the aminopolyamines. At each of different time points, OD_{660} was recorded and 100 μL of the bacteria suspension was withdrawn and diluted to 10^{-7}, 10^{-8}, and 10^{-9}. One hundred microliters of each of these diluted bacteria suspension was placed on the agar plates. The agar plates were then incubated at 30 ^{0}C for 24-48 h and counted for CFU.

Polyamine Content Analysis

The bacteria were grown in nutrient broth with or without drugs. Culture samples were collected between late logarithmic and stationary phases and centrifuged at 4000 rpm for 20 min. The pellet was washed twice with phosphate buffered saline solution before resuspending in 1.5 mL of phosphate buffer and 1.5 mL of 1.2 N $HClO_4$. The suspension was subjected to three freeze-thaw cycles and centrifuged at 4500 rpm for 20 min to separate the cell debris. The supernatant containing polyamines was used for polyamine content analyses.

Analytical Development

According to the method of Kallio and Bitonti, bacteria were first harvested by centrifugation. The pellets were washed once with 10 mM Na_2PO_4/ 3 mM KH_2PO_4/ 125mM NaCl (pH 7.2) and extracted overnight with 0.4 *N* $HClO_4$ (Bitonti, 1984). The suspension was then filtered through a 0.22 μm Millipore membrane to remove proteins and other cellular debris. The supernatant was analyzed for polyamines with a Dionex D-300 Polyamine Analyzer using a Dionex P/N 30831 cation-separator column followed by post-column derivatization with *o*-phthalaldehyde and fluorescence detection (Kallio, 1982).

Based on our experience with dansylation as an analytical method for quantitating natural polyamines, their analogs and their corresponding metabolites (Bergeron et al., 1995, 1997, and 2000), we extended this method to the aminopolyamine series. The HPLC system utilized a 4.6 x 250 mm Symmetry C18 column with a C18 guard column. The system was also equipped with a Shimadzu RF-10Ax1 fluorescence detector with excitation and emission wavelengths of 350 nm and 509 nm, respectively. Mobile phase

A was 93% aqueous methanol and B was 65% aqueous acetonitrile. The flow rate was 1.5 mL/min and the gradients were set as described in Table 6-1. The resulting retention times of the DANSYL derivatives are shown in Table 6-2. The calibration curves for the dansylated derivatives of natural polyamines and aminopolyamines are reported (Fig. 6-2 to Fig. 6-8). The linear coefficient value (R^2) was at least 0.996.

Dansylation Procedure

To a 100 μL supernatant containing polyamines and their metabolites, 120 mg solid anhydrous Na_2CO_3, 30 μL of 1.25 x 10^{-4} M 1,6-diaminohexane (internal standard) in 0.6 N HCl, and 400 μL of a 5 mg/mL dansyl chloride solution in dry acetone were added. After mixing, the vial (protected from light from this point on) was placed in water bath at 70 ^{0}C for 20 min and then cooled to room temperature. Excess dansyl chloride was reacted with 100 μL of 250 mg/mL L-proline in water. The mixture was vortexed thoroughly and incubated at room temperature for 10 min. Five hundred microliters of 25% aqueous acetonitrile were added to the dansylated mixture. The solution was vortexed, and applied to a C18 SPE column (washed with methanol then 25% aqueous acetonitrile 18 mL each). Two additional 500-μL portions of 25% aqueous acetonitrile were added to the dansylated mixture and applied to a C18 SPE column. After application of vacuum, the columns were washed twice with 25% aqueous acetonitrile (2 x 2.5 mL). Each sample was eluted with two separate 2 mL aliquots of methanol. One hundred microliters of this solution containing dansylated polyamine was injected to HPLC for polyamine analysis. Figure 6-1 shows the dansylation reaction of 7-NH_2-SPD with dansyl chloride in the presence of sodium carbonate in acetone to form a tetradansylated-7-aminospermidine derivative. Dansyl is a fluorescent labeling group.

Syntheses

All chemical reagents and solvents were purchased from Aldrich Chemical Co., Fisher Scientific, and Fluka. DMF was routinely distilled and stored over molecular sieves. Fisher Optima grade solvents were used and sodium sulfate was used to dry the organic extracts. Silica gel 32-63 mesh from Selecto, Inc. (Kennesaw, GA) was used for flash column chromatography. Nuclear magnetic resonance (NMR) spectroscopy was carried out on a Varian Unity 300. Proton NMR spectra were run at 300 MHz in $CDCl_3$ (not specified) or D_2O with chemical shifts in parts per million downfield from tetramethylsilane (TMS) or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP), respectively. ^{13}C NMR spectra were recorded at 75 MHz with dioxane (δ 67.19) as reference. Coupling constants (J) are in hertz. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

Synthesis of 2-Aminoputrescine

Mixture of N^1- and N^2-Formyl-N^1,N^2,N^4-tris-(*tert*-butoxycarbonyl)-1-butene-1,2,4-triamines (2): A mixture of **1** (4.00 g, 21.7 mmol), $NaHCO_3$ (4.00 g, 44 mmol), di-*tert*-butyl dicarbonate (27.0 g, 124 mmol) in acetonitrile (100 mL) and 15% potassium acetate (100 mL) was stirred at room temperature for 5 d. The organic layer was separated and concentrated under reduced pressure. The oily residue was taken up in EtOAc, washed with water, brine and concentrated. Purification by chromatography eluting with 2:1 hex/EtOAc gave **2** as a mixture (6.23 g, 66%). ^1H NMR δ 9.2 (s, 1 H, aldehyde) common for the 2 isomers.

N^1,N^2,N^4-Tris-(*tert*-butoxycarbonyl)-1-butene-1,2,4-triamine (3): A solution of **2** (6.4 g, 14.9 mmol) in MeOH (150 mL) and heated at reflux for 3 h. The resulting

solution was cooled to room temperature and concentrated to obtain a viscous oil which was recrystalized from EtOAc/hex to afford (**3**) a white foam (5.94 g, 91%). ^{1}H NMR δ 1.40-1.50 (3 singlets, 27 H), 2.24 (t, 2 H, J = 6), 3.19 (q, 2 H, J = 6), 4.78 (broad s, 1 H), 5.93 (broad s, 1 H), 6.24 (d, 1 H, J = 10).

N^1,N^2,N^4-Tris-*tert*-(butoxycarbonyl)butane-1,2,4-triamine (4): Palladium (10% in activated carbon, 0.45 g) was added to a solution of **3** (4.21 g, 10.5 mmol) in EtOH (50 mL). The reaction mixture was stirred under H_2 at atmospheric pressure and at 55 ^{0}C for 17 h. The solution was filtered through celite and concentrated under reduced pressure to give **4** (3.81g, 90%) as a white foam. ^{1}H NMR 1.44 (s, 27 H), 3.00 (m, 1 H), 3.2 (m, 2 H), 3.4 (m, 1 H), 3.7 (m, 1 H), 4.9 (m, 2 H).

2-Aminoputrescine trihydrochloride (5): Dry HCl (20% in EtOH, 20 mL) was added to a solution of **4** (1.20 g, 2.97 mmol) in dry EtOH (10 mL). The solution was stirred at room temperature for 12 h. A white solid was formed. Ether was then added to form more solid. The solid was filtered and recrystallized from aqueous EtOH to obtain **5** (0.581 g, 92%) as a white solid. ^{1}H NMR (D_2O) δ 2.20 (m, 2 H), 3.20-3.25 (m, 2 H), 3.40 (m, 2 H), 3.78 (quintet, 1 H, J = 6). HRMS *m/z* cald for $C_4H_{13}N_3$ (free amine) 104.0 [M+1], found 104.0; Anal. Cald for $C_4H_{16}Cl_3N_3$: C, 22.60; H, 7.59; N, 19.77. Found: C, 22.76; H, 7.65; N, 19.65.

Synthesis of 7-Aminospermidine

N^1, N^4-Bis(mesitylenesulfonyl)histamine (6): Mesitylenesulfonyl chloride (20.7 g, 94.0 mmol) in CH_2Cl_2 (300 mL) was added dropwise with stirring to histamine dihydrochloride (**1**) (8.00 g, 44.0 mmol) in 1 *N* NaOH (180 mL) at 0 ^{0}C. The mixture was then stirred for 20 h at room temperature. The layers were separated, and the

aqueous layer was further extracted with CH_2Cl_2 (4 x 50 mL). The combined organic layers were washed with 1 *N* NaOH, 1 *N* HCl, H_2O, brine and concentrated under reduced pressure. Recrystallization with aqueous EtOH produced a white solid **6** (19.8 g, 95%). ^{1}H NMR δ 2.05 (s, 6 H), 2.30 (s, 6 H), 2.50 (s, 6 H), 3.45-3.65 (m, 4 H), 6.75 (s, 2 H), 6.8 (s, 1 H), 7.1 (s, 2 H), 7.9 (s, 1 H); HRMS *m/z* calcd for $C_{23}H_{30}N_3O_4S_2$ 476.1599 [M+H], found 476.1675.

***N*-propylphthalimide-N^1,N^4-bis(mesitylenesulfonyl)histamine (7)**: Sodium hydride (60%, 2.00 g, 50 mmol) was added carefully to an ice-cooled solution of **6** (19.41 g, 41 mmol) in DMF (200 mL). The mixture was stirred at room temperature for 1 h followed by the addition of *N*-(3-bromopropyl)phthalimide (13.52 g, 50.4 mmol) in DMF (100 mL). After stirring overnight at room temperature, the reaction mixture was heated to 70 ^{0}C and stirred for 4 h. The resulting mixture was cooled to 0 ^{0}C, quenched with water, and concentrated under high vacuum. The residue was then taken up in CH_2Cl_2 (300 mL) and water (100 mL), and the layers were separated. The aqueous layer was extracted with chloroform (3 x 100 mL). The combined organic layers were washed with water, brine and concentrated under reduced pressure. Flash chromatography eluting with 1:1 hex/EtOAc gave **7** (12 g, 45%) as viscous oil. ^{1}H NMR δ 1.61-1.69 (m, 2 H), 2.05 (s, 6 H), 2.45 (s, 6 H), 2.6 (s, 6 H), 2.8 (m, 2 H), 3.15-3.20 (m, 2 H), 3.45-3.55 (m, 4 H), 6.79 (s, 2 H), 6.82 (s, 1 H), 7.0 (s, 2 H), 7.8 (s, 1 H), 7.75-7.80 (m, 5 H). ^{13}C NMR δ 168.01, 142.81, 140.03, 139.33, 138.72, 136.96, 133.93, 131.97, 130.96, 130.67(d), 123.18, 116.30, 45.14, 43.90, 35.07, 26.45, 23.81, 22.93, 22.68, 20.89, 20.74; HRMS *m/z* calcd for $C_{25}H_{29}N_4O_4S$ [M-Mts] 481.1988 found 481.1907; Mts = mesitylenesulfonyl, $C_9H_{11}O_2S$.

***N*-(3-Aminopropyl)-N^1,N^4-bis(mesitylenesulfonyl)histamine (8)**: Hydrazine monohydrate (1.8 g, 36 mmol) was added to a solution of **7** (12 g, 18.1 mmol) in EtOH (175 mL). The reaction mixture was heated to reflux for 2 h. After cooling the solution to room temperature, phthalhydrazide solid was filtered and the filtrate was evaporated under reduced pressure. Flash column chromatography eluting with 4% NH_4OH/MeOH gave **8** (5.4 g, 56%) as a viscous oil. ^{1}H NMR δ 1.63 (quin, 2 H, J = 6), 2.3 (s, 6 H), 2.5 (s, 6 H), 2.6 (s, 6 H), (2.8, m, 2 H), 2.73 (t, 2 H, J = 7), 3.2 (t, 2 H, J = 7), 3.4 (t, 2 H, J = 7), 6.68 (s, 1 H), 6.90 (s, 2 H), 6.92 (s, 2 H), 7.52 (s, 1 H). HRMS *m/z* cald for $C_{26}H_{37}N_4O_4S_2$, 533.2178 [M+H], found 533.2229.

***N*-(3-Aminopropyl)histamine trihydrobromide (9)**: Hydrogen bromide in HOAc (30%, 80 mL) was added dropwise to a solution of **8** (5.4 g, 10 mmol) and phenol (37.6 g, 0.400 mol) in CH_2Cl_2 (120 mL) at 0 ^{0}C. The mixture was stirred overnight at room temperature. Water (150 mL) was added followed by extraction with CH_2Cl_2 (3 x 100 mL). The aqueous layer was evaporated under high vacuum. Recrystallization from aqueous EtOH gave **9** as a white solid (2.7 g, 66%). ^{1}H NMR (D_2O) δ2.1 (m, 2 H), 3.1 (t, 2 H, J = 7.5), 3.2 (m, 4 H), 3.45 (t, 2 H, J = 7.5), 7.4 (s, 1 H), 8.7 (s, 1 H); ^{13}C (D_2O, Dioxane, 67.19) δ21.30, 23.83, 36.64, 44.85, 46.02, 117.12, 128.19, 134.06. HRMS *m/z* calcd for $C_8H_{17}N_4$ [M+1] (free amine), 169.1375, found 169.1450.

Mixture of N^1-and N^2-formyl-N^1,N^2,N^4-tris-(*tert*-butoxycarbonyl)-N^4-(N-*tert*-butoxycarbonyl-3-aminopropyl)-1-butene-1,2,4-triamines (10): A mixture of **9** (2.8 g, 7.0 mmol), $NaHCO_3$ (2.52 g, 30 mmol), di-*tert*-butyl dicarbonate (12.23 g, 56 mmol) in acetonitrile (60 mL) and 15% potassium acetate (30 mL) was stirred at room temperature for 5 d. The organic layer was separated and concentrated under reduced pressure. The

oily residue was taken up in EtOAc, washed with water, brine and concentrated. Purification by chromatography eluting with 2:1 Hex/EtOAc gave **10** as a mixture (3.01 g, 73%). ^{1}H NMR δ 9.2 (s, 1 H, aldehyde) common for the 2 isomers; ^{13}C δ 154.11, 152.11.

N^1,N^2,N^4-Tris(*tert*-butoxycarbonyl)-N^4-(*N*-*tert*-butoxycarbonyl-3-aminopropyl)-1-butene-1,2,4-triamines (11): A solution of **10** (2.01 g, 3.41 mmol) in MeOH (100 mL) was heated at reflux for 3 h. The resulting solution was cooled to room temperature and concentrated to obtain a white foam (**11**) (1.91 g, 95%) which was used directly in the next step. ^{1}H NMR δ 1.40-1.45 (s, 36 H), 1.60 (m, 4 H), 1.65 (t, 1 H, J = 6), 2.3 (t, 1 H, J = 6), 3.00-3.30 (m, 4 H), 6.15 (d, 1 H, J = 9).

N^1,N^2,N^4-Tris(*tert*-butoxycarbonyl)-N^4-(*N*-*tert*-butoxycarbonyl-3-aminopropyl)-butane-1,2,4-triamines (12): Palladium (10% in activated carbon, 0.38 g) was added to a solution of **11** (1.91 g, 3.42 mmol) in EtOH (100 mL). The reaction mixture was stirred under H_2 at atmospheric pressure and at 49 ^{0}C for 17 h. The solution was filtered through celite and concentrated under reduced pressure to give **12** (1.8 g, 95%) as a white foam. ^{1}H NMR 1.42-1.48 (s, 36 H), 1.65 (m, 4 H), 3.0-3.3 (m, 8 H), 3.6 (m, 1 H). HRMS *m/z* calcd for $C_{27}H_{53}N_4O_8$, 561.38 [M+H], found 561.38.

7-Aminospermidine tetrahydrochloride (13): Dry HCl (20% in EtOH, 50 mL) was added to a solution of **12** (1.8 g, 3.21 mmol) in dry EtOH (50 mL). The solution was stirred at room temperature for 12 h. A white solid was formed. Ether was then added to form more solid. The solid was filtered and recrystallized from aqueous EtOH to obtain **13** (0.97 g, 98%) as a white solid. ^{1}H NMR (D_2O) δ 2.1-2.3 (m, 4 H), 3.1 (t, 2 H, J = 8), 3.22 (t, 2 H, J = 8), 3.25-3.35 (m, 2 H), 3.35-3.45 (m, 2 H), 3.74-3.85 (m, 1 H); ^{13}C (D_2O,

Dioxane, 67.19) δ24.00, 27.33, 36.73, 40.90, 43.80, 45.06, 47.43. HRMS *m/z* cald for $C_7H_{21}N_4$ (free amine) 161.1688 [M+1], found 161.1765; Anal. Calcd for $C_7H_{24}Cl_4N_4$: C, 27.41; H, 7.90; N, 18.30. Found: C, 27.51; H, 7.88; N, 18.19.

Synthesis of N^1,N^8-Diethyl-7-Aminospermidine

N^1,N^3-Bis(mesitylenesulfonyl)-3-ethylaminopropylamine (15): prepared according to the procedure of Bergeron et al. 2001b.

N^1-(3,4-epoxybutyl)-N^1,N^3-bis(mesitylenesulfonyl)-3-ethylaminopropylamine (16): A solution of **15** (69 g, 0.148 mol) in DMF (700mL) was cooled to 0 ^{0}C under N_2 atmosphere. Sodium hydride (60%, 6.84 g, 0.171 mol) was carefully added. The reaction mixture was stirred at room temperature for 1.5 h followed by the addition of 1-bromo-3,4-epoxybutane (23.5 g, 0.155 mol) in DMF (200 mL). The reaction mixture was stirred at room temperature overnight. Water (50 mL) was then added and the mixture was concentrated under high vacuum. The crude solid was taken up in $CHCl_3$ (300 mL) and H_2O (200 mL) and the two layers were separated. The aqueous layer was extracted with $CHCl_3$ (2 x 100 mL). The combined chloroform layers were washed with H_2O (200 mL), brine (400 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Flash column through silica gel eluted by 3:2 hexane/EtOAc gave **16** (77 g, 90%) as a viscous oil. ^{1}H NMR δ0.95 (t, 3 H, J = 7), 1.5-1.8 (m, 4 H), 2.3 (s, 6 H), 2.4 (m, 1 H), 2.56 (s, 12 H), 3.0-3.4 (m, 8 H), 6.95 (s, 4 H), 2.65 (m, 1 H), 2.75 (m, 1 H); HRMS *m/z* calcd for $C_{27}H_{41}N_2O_5S_2$ [M+H] 537.2379, found 537.2477.

N^1,N^8-Diethyl-N^1,N^4,N^8-tris(mesitylenesulfonyl)-7-hydroxyspermidine (17): Sodium hydride (60%, 1.8 g, 45 mmol) was added slowly to a solution of N-ethylmesitylenesulfonamide (9.3 g, 40.8 mmol) in DMF (300 mL) at 0 ^{0}C. The mixture

was stirred at room temperature for 45 min followed by the addition of epoxide **16** (21.9 g, 40.8 mmol) in DMF (100 mL). The reaction mixture was stirred at 70 ^{0}C overnight. Water (50 mL) was added and the mixture was concentrated under high vacuum. The crude product was taken up in water (250 mL) and the resulting mixture was extracted with $CHCl_3$ (3 x 300 mL). The combined organic layers were washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash chromatography on silica gel eluted with 2:1 hexane/EtOAc yielded **17** (20.54 g, 66%) as a viscous oil. ^{1}H NMR δ0.95 (t, 3 H, J = 7), 1.03 (t, 3 H, J = 7), 1.60-1.70 (m, 4 H), 2.30 (s, 9 H), 2.53-2.60 (s, 18 H), 3.05-3.15 (m, 12 H), 3.35-3.45 (m, 1 H), 6.90 (s, 2 H), 6.92 (s, 2 H), 6.96 (s, 2 H); HRMS *m/z* calcd for $C_{38}H_{58}N_3O_7S_3$ [M+H] 764.3359, found 764.3462.

N^1,N^8-Diethyl-N^1,N^4,N^8-tris(mesitylenesulfonyl)-7-*O*-(tosyloxy)spermidine (18): A solution of **17** (20.5 g, 26.8 mmol) in anhydrous pyridine (80 mL) was cooled to 0 ^{0}C. *p*-Toluenesulfonyl chloride (10.22 g, 53.6 mmol) in anhydrous CH_2Cl_2 (60 mL) was added dropwise and the reaction mixture was stirred at room temperature overnight. The resulting mixture was diluted with $CHCl_3$ (500 mL) then poured into a slurry of ice (200 g) and 1 N HCl (400 mL). The organic layer was separated, washed with water (200 mL), saturated $NaHCO_3$ solution (200 mL), brine (300 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by silica flash column eluting with 2:1 Hex/EtOAc to afford **18** (18.5 g, 75%) as a foam. ^{1}H NMR δ0.85-1.00 (m, 6 H), 1.60 (m, 2 H), 2.80-3.20 (m, 12 H), 3.4 (d, 2 H, J = 5.4), 4.40 (quin, 1 H, J = 5.4), 6.90-6.96 (s, 6 H), 7.36 (d, 2 H, J = 8), 7.76 (d, 2 H, J = 8); HRMS m/z calcd for $C_{45}H_{64}N_3O_9S_4$ [M+H] 918.3447, found 918.3564.

N^1, N^8-Diethyl-N^1, N^4, N^8-tris(mesitylenesulfonyl)-7-(phthalimido)spermidine (19): Potassium phthalimide (5.6 g, 30.2 mmol) in DMF (100 mL) was added to a solution of **18** (18.5 g, 20.1 mmol) in DMF (200 mL). The reaction mixture was stirred at 85 ^{0}C overnight. The cooled reaction mixture was concentrated under high vacuum. The residue was taken up in water (300 mL) and the aqueous solution was extracted with $CHCl_3$ (3 x 200 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Flash column eluted with 12:1 $CHCl_3$/EtOAc then 10:1 $CHCl_3$/EtOAc yielded **19** (17.1 g. 90%) as a white foam. ^{1}H NMR δ 0.92 (t, 3 H, J = 7), 1.15 (t, 3 H, J = 7), 1.6 (m, 2 H), 2.3 (s, 9 H), 2.4 (s, 9 H), 2.5 (s, 9 H), 2.85-3.20 (m, 14 H), 3.78-3.83 (m, 1 H), 6.6 (s, 2 H), 6.8 (s, 2 H), 6.9 (s, 2 H), 7.65-7.75 (m, 4 H); HRMS *m/z* calcd for $C_{46}H_{61}N_4O_8S_3$ [M+1] 893.3573, found 893.3677.

N^1, N^8-Diethyl-N^1,N^4,N^8-tris(mesitylenesulfonyl)-7-aminospermidine (20): Sodium borohydride (2.74 g, 72.5 mmol) was added to a stirred solution of **19** (13.0 g, 14.5 mmol) in CH_2Cl_2 (30 mL), 2-propanol (200 mL) and water (33.6 mL). The reaction mixture was stirred at room temperature overnight. After running TLC (4:1 chlorofom/EtOAc) to make sure **19** had completely reacted, glacial acetic acid (24 mL) was added carefully (pH ~ 5). When the foaming subsided, the reaction mixture was stirred at 80 ^{0}C overnight. The resulting mixture was concentrated under reduced pressure. The residue was taken up in water and extracted with $CHCl_3$ (6 x 100 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution, water, brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Flash column chromatography eluting with 4:1 $CHCl_3$/EtOAc then 1:1 $CHCl_3$/EtOAc gave **20** (6.01g,

55%) as a white foam. ^{1}H NMR δ0.94 (t, 3 H, J = 7), 1.02 (t, 3 H, J = 7), 1.73 (m, 3 H), 2.25 (s, 9 H), 2.55 (s, 18 H), 2.95-3.3 (m, 13 H), 3.6 (m, 1 H), 6.85-6.90 (s, 6 H); HRMS *m/z* calcd for $C_{38}H_{59}N_4O_6S_3$ [M+H] 763.3518, found 763.3604.

***N*^{1}, *N*^{8}-Diethyl-7-aminospermidine tetrahydrochloride (21)**: Hydrogen bromide in HOAc (30%, 160 mL) was added slowly to a solution of **20** (6.01 g, 7.9 mmol) and phenol (30 g, 319 mmol) in CH_2Cl_2 (100 mL) at 0 ^{0}C. The suspension was stirred overnight at room temperature. Water was added followed by extraction with CH_2Cl_2 (3 x 150 mL). The aqueous layer was evaporated under high vacuum, and the residue was taken up in 1 N NaOH (7 mL) and 19 N NaOH (6 mL) (pH > 14) followed by extraction with $CHCl_3$ (10 x 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The oily residue was taken up in EtOH (75 mL) and acidified with concentrated HCl (6 mL). After the solvent was removed, the solid was recrystallized from aqueous EtOH to give **21** (1.4 g, 50%) as a white powder. ^{1}H NMR (D_2O), δ1.31 (t, 3 H, J = 7), 1.35 (t, 3 H, J = 7), 2.10-2.30 (m, 4 H), 3.10-3.40 (m, 12 H), 3.86 (quin, 1 H, J = 7). HRMS *m/z* calcd for $C_{11}H_{29}N_4$ [M+H] (free amine) 217.2314, found 217.2373; Anal. Calcd. for $C_{11}H_{33}N_4Cl_4$: C, 36.48; H, 8.90; N, 15.47. Found: C, 36.00; H, 8.89; N, 15.19.

Synthesis of 6-Aminospermine

***N*, *N*'-bis(mesitylenesulfonyl)-*N*-benzyl-1,3-diaminopropane (22)**: A solution of mesitylenesulfonyl chloride (14.80 g, 67.0 mmol) in CH_2Cl_2 (100 mL) was added dropwise to a rapidly stirred mixture of 3-benzylaminopropylamine (**41**)(5.00 g, 30.4 mmol) in 1.0 *N* NaOH (70 mL) at 0 ^{0}C. After the addition was completed, the mixture was stirred at room temperature for 30 h. Upon completion of the reaction, the organic layer was separated while the aqueous layer (top layer) was extracted with chloroform (3

x 75 mL). The combined organic layers were washed with 1.0 *N* HCl (100 mL), water (100 mL), brine (150 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The yellow viscous oil obtained was then purified by silica gel chromatography eluting with 2:1 hexane/EtOAc to afford white foam (**22**) (8.41 g, 60%). ^{1}H NMR δ 1.53-1.63 (quin, 2 H, J = 7), 2.27 (s, 3 H), 2.32 (s, 3 H), 2.60 (s, 12 H), 2.80-2.90 (m, 2 H), 3.18 (t, 2 H, J = 7), 4.2 (s, 2 H), 6.92 (s, 2 H), 6.96 (s, 2 H), 6.98-7.02 (m, 2 H), 7.20-7.28 (m, 3 H); HRMS *m/z* calcd for $C_{28}H_{37}N_2O_4S_2$ [M+H] 529.2116, found 529.2183.

N^1-(3, 4-Epoxybutyl)-N^1, N^3-bis(mesitylenesulfonyl)-3-benzylaminopropylamine (23): A solution of **22** (8.40 g, 15.9 mmol) in DMF (83 mL) was cooled to 0 ^{0}C under N_2. NaH (60%, 0.764 g, 19.1 mmol) was carefully added. The reaction mixture was stirred at room temperature for 1.0 h followed by the addition of 1-bromo-3, 4-epoxybutane (4.84 g, 32.1 mmol) in DMF (25 mL). The reaction mixture was stirred at room temperature overnight. Ice (10 g) was then added to quench the reaction and the mixture was concentrated under high vacuum. The crude yellow viscous oil was taken up in $CHCl_3$ (100 mL) and H_2O (100 mL) and the two layers were separated. The aqueous layer was extracted with $CHCl_3$ (2 x 80 mL). The combined chloroform layers were washed with H_2O (100 mL), brine (200 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Flash column through silica gel eluting with 2:1 hexane/EtOAc gave **23** (7.53 g, 79%) as a white foam. ^{1}H NMR δ 1.45-1.75 (m, 5 H), 2.25 (s, 3 H), 2.30 (s, 3 H), 2.50 (s, 6 H), 2.60 (s, 6 H), 2.65-2.75 (m, 2 H), 2.85 (t, 2 H, J = 9), 2.95 (t, 2 H, J = 9), 3.15-3.25 (m, 2 H), 4.20 (s, 2

H), 6.90 (s, 2 H), 6.95 (s, 2 H), 7.05 (m, 2 H), 7.25 (m, 3 H); HRMS *m/z* calcd for $C_{32}H_{42}N_2NaO_5S_2$ [M+Na] 621.2535, found 621.2417.

N^1, N^{12}-Dibenzyl-N^1, N^4, N^9, N^{12}-tetrakis(mesitylenesulfonyl)-6-hydroxyspermine (24): Sodium hydride (60%, 1.96 g, 49.0 mmol) was added slowly to a solution of **22** (21.55 g, 40.8 mmol) in DMF (300 mL) at 0^0C. The mixture was stirred at room temperature for 45 min followed by the addition of epoxide **23** (21.9 g, 40.8 mmol) in DMF (100 mL). The reaction mixture was stirred at 75 ^{0}C overnight. Water (50 mL) was added and the mixture was concentrated under high vacuum. The crude product was taken up in water (250 mL) and the resulting mixture was extracted with $CHCl_3$ (3 x 300 mL). The combined organic layers were washed with water, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash chromatography eluting with 2:1 hex/EtOAc yielded **24** (20.54 g, 66%) as a viscous oil. ^{1}H NMR δ0.95 (t, 3 H, J = 7), 1.03 (t, 3 H, *J* = 7), 1.60-1.70 (m, 4 H), 2.30 (s, 9 H), 2.53-2.60 (s, 18 H), 3.05-3.15 (m, 12 H), 3.35-3.45 (m, 1 H), 6.90 (s, 2 H), 6.92 (s, 2 H), 6.96 (s, 2 H); HRMS *m/z* calcd for $C_{38}H_{58}N_3O_7S_3$ [M+H] 764.3359, found 764.3462.

N^1, N^{12}-Dibenzyl-N^1, N^4, N^9, N^{12}-tetrakis(mesitylenesulfonyl)-6-*O*-(tosyloxy)spermine (25): A solution of **24** (2.10 g, 1.86 mmol) in anhydrous pyridine (6.55 mL, 81.4 mmol) was cooled to 0 ^{0}C. *p*-Toluenesulfonyl chloride (0.709 g, 3.72 mmol) in anhydrous CH_2Cl_2 (15 mL) was added dropwise and the reaction mixture was stirred at room temperature overnight. The resulting mixture was diluted with $CHCl_3$ (50 mL) then poured into a slurry of ice (100 g) and 1 *N* HCl (300 mL). The organic layer was separated, washed with water (100 mL), saturated $NaHCO_3$ solution (100 mL), brine (150 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced

pressure. The crude product was purified by silica gel flash column eluting with 2:1 Hex/EtOAc to afford **25** (1.72 g, 88%) as a white foam. ^{1}H NMR δ 1.50-1.70 (m, 7 H), 2.28 (d, 6 H, J = 1.5), 2.32 (d, 6 H, J = 1.5), 2.42 (s, 15 H), 2.58 (s, 12 H), 2.62-2.90 (m, 11 H), 2.90-3.07 (m, 1 H), 4.18 (s, 2 H), 4.20 (s, 2 H), 6.90 (d, 4 H, J = 9), 6.96 (d, 4 H, J = 4.5), 6.99-7.06 (m, 4 H), 7.20-7.30 (m, 6 H), 7.35 (d, 2 H, J = 8), 7.76 (d, 2 H, J = 8); HRMS *m/z* calcd for $C_{67}H_{84}N_4NaO_{11}S_5$ [M+Na] 1303.4740, found 1303.4632.

N^1, N^{12}-Dibenzyl-N^1, N^4, N^9, N^{12}-tetrakis(mesitylenesulfonyl)-6-(phthalimido)-spermine (26): Potassium phthalimide (1.53 g, 8.26 mmol) was added to a solution of **25** (5.30 g, 4.13 mmol) in DMF (60 mL). The reaction mixture was stirred at 90 ^{0}C overnight. The next day, the resulting mixture was concentrated under high vacuum. The residue was taken up in water (200 mL) and the aqueous solution was extracted with $CHCl_3$ (3 x 100 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Flash column eluting with 12:1 $CHCl_3$/EtOAc then 10:1 $CHCl_3$/EtOAc yielded **26** (2.81 g, 54%) as a white foam. ^{1}H NMR δ 1.5-1.8 (m, 7 H), 2.25-2.65 (all singlets, 36 H), 2.70-3.20 (m, 11 H), 3.75-3.82 (m, 1 H, CH-Phthalimide), 4.10-4.20 (m, 4 H), 6.5-7.7 (m, 22 aromatic H's); HRMS *m/z* calcd for $C_{68}H_{81}N_5NaO_{10}S_4$ [M+Na] 1278.4866, found 1278.4758.

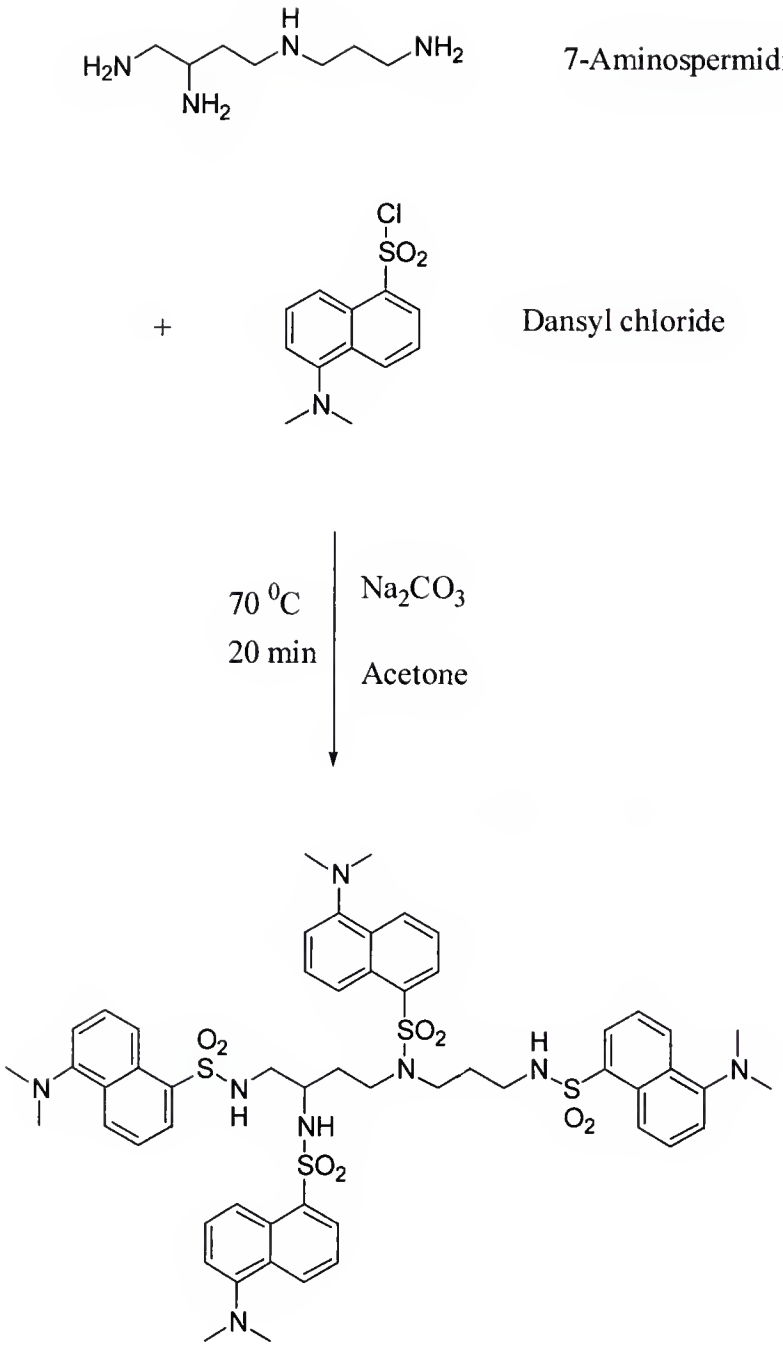
N^1, N^{12}-Dibenzyl-N^1, N^4, N^9, N^{12}-tetrakis(mesitylenesulfonyl)-6-aminospermine (27): Sodium borohydride (0.567 g, 15.0 mmol) was added to a stirred solution of **26** (2.81 g, 2.24 mmol) in CH_2Cl_2 (10 mL), 2-propanol (40 mL) and water (7 mL). The reaction mixture was stirred at room temperature overnight. After running TLC (2:1 chlorofom/EtOAc) to make sure **26** had completely reacted, glacial acetic acid (4.5 mL) was added carefully (pH ~ 5). When the foaming subsided, the reaction mixture

was stirred at 80 ^{0}C overnight. The resulting mixture was heated for additional 4 h before concentrating under reduced pressure. The residue was taken up in water and extracted with $CHCl_3$ (6 x 50 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution, water, brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Flash column eluting with 1:1 $CHCl_3$/EtOAc gave **27** (1.59 g, 63%) as a white foam. ^{1}H NMR δ 1.3-1.7 (m, 7 H), 2.28, 2.31, 2.49, 2.50, 2.57 (all singlets, 36 H), 2.75-3.00 (m, 12 H), 4.05-4.25 (m, 4 H), 6.90-7.25 (m, 18 aromatic H's); HRMS m/z calcd for $C_{60}H_{80}N_5O_8S_4$ [M+H] 1126.4811, found 1126.4884.

***N*^{1}, *N*^{12}-Dibenzyl-6-aminospermine (28)**: Hydrogen bromide in HOAc (30%, 25 mL) was added slowly to a solution of **27** (1.58 g, 1.40 mmol) and phenol (5.26 g, 56 mmol) in CH_2Cl_2 (10 mL) at 0 ^{0}C. The suspension was stirred overnight at room temperature. Water was added followed by extraction with CH_2Cl_2 (3 x 150 mL). The aqueous layer was evaporated under high vacuum, and the residue was taken up in 1 N NaOH (1 mL) and 19 N NaOH (1 mL) (pH > 14) followed by extraction with $CHCl_3$ (10 x 15 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The oily residue **28** (as a free base) was used directly in the next reaction.

6-Aminospermine pentahydrochloride (29): A solution of the oily residue **28** (0.630 g, ~ 1.6 mmol) and Pd/C (0.120 g) in 1 *N* HCl (8.0 mL, 8.0 mmol) and EtOH (50 mL) was stirred under H_2 at 1 atm overnight. The reaction mixture was then slowly heated to 55 ^{0}C under H_2 for another day. After filtration through celite, the solvent was evaporated and the yellow solid was recrystallized from aqueous EtOH to afford **29** as a white solid (28.3 mg, 60% yield from **27**). ^{1}H NMR δ 2.06-2.32 (m, 6 H), 3.08-3.35 (m,

12 H), 3.82 (quintet, 1 H, J = 6.6); HRMS *m/z* calcd for $C_{10}H_{28}N_5$ [M+H] 218.2266, found 218.2353.



Tetradansylated-7-aminospermidine

Figure 6-1: Dansylation reaction of 7-aminospermidine
(Assuming tetradansylation occured for 7-aminospd)

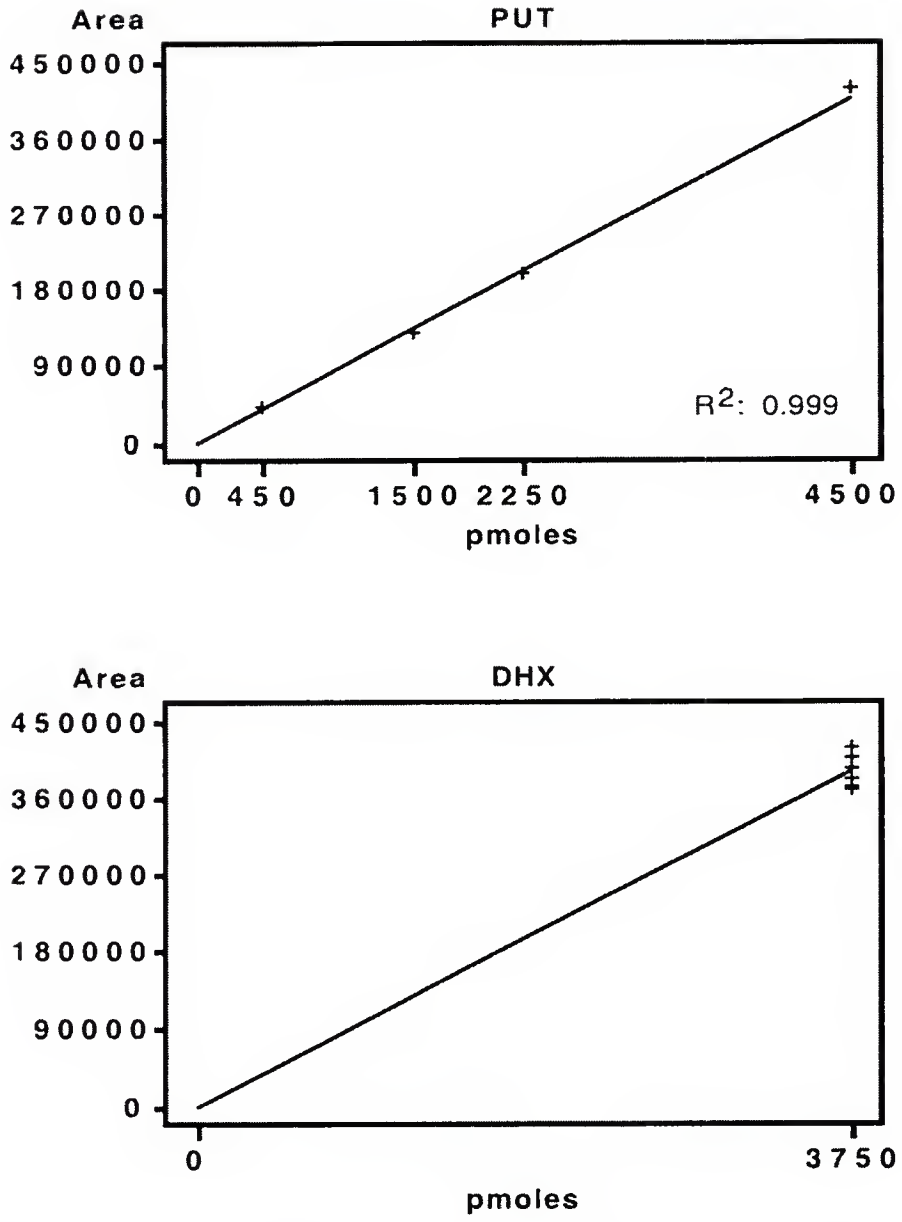


Figure 6-2. HPLC calibration curve for PUT and DHX (diaminohexane, internal standard). Each plot shows the amount (in picomoles) of the polyamine or the analogue versus the area under the curve.

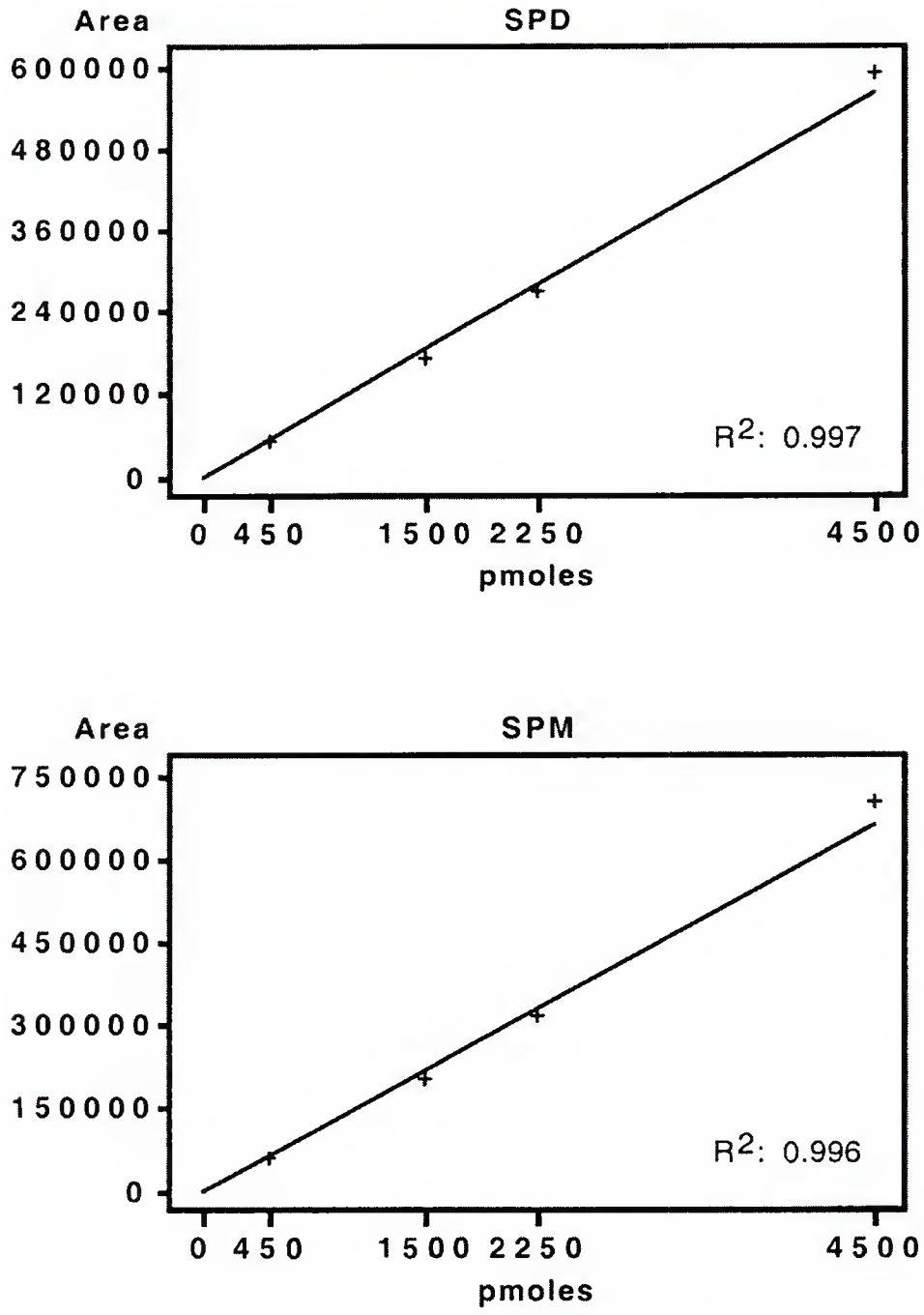


Figure 6-3. HPLC calibration curve for SPD and SPM. Each plot shows the amount (in picomoles) of the polyamine or the analogue versus the area under the curve.

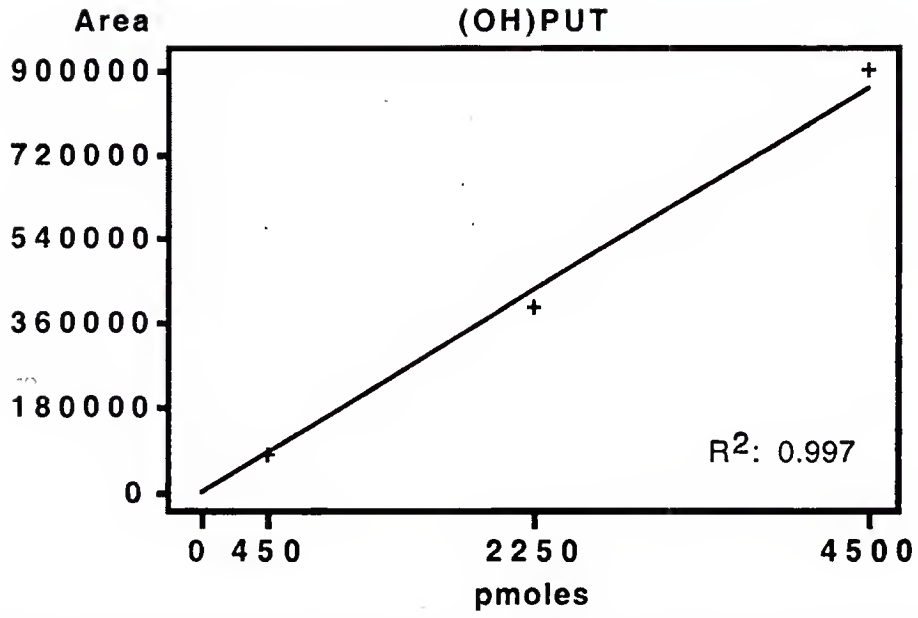


Figure 6-4. HPLC calibration curve for 2-OH-PUT (also known as (OH)PUT). The plot shows the amount (in picomoles) of 2-OH-PUT versus the area under the curve.

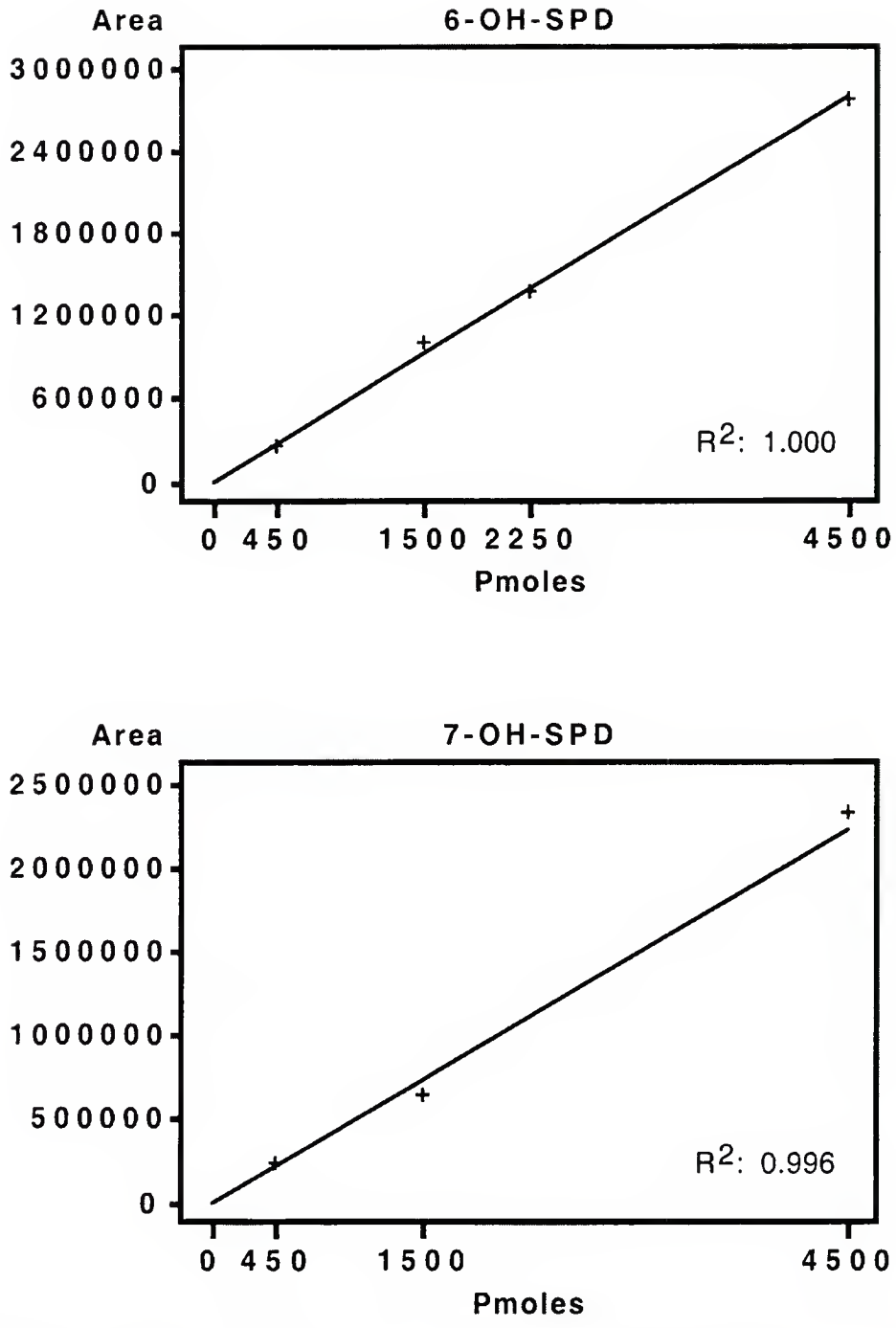


Figure 6-5. HPLC calibration curve for 6-OH-SPD and 7-OH-SPD. Each plot shows the amount (in picomoles) of each analogue versus the area under the curve.

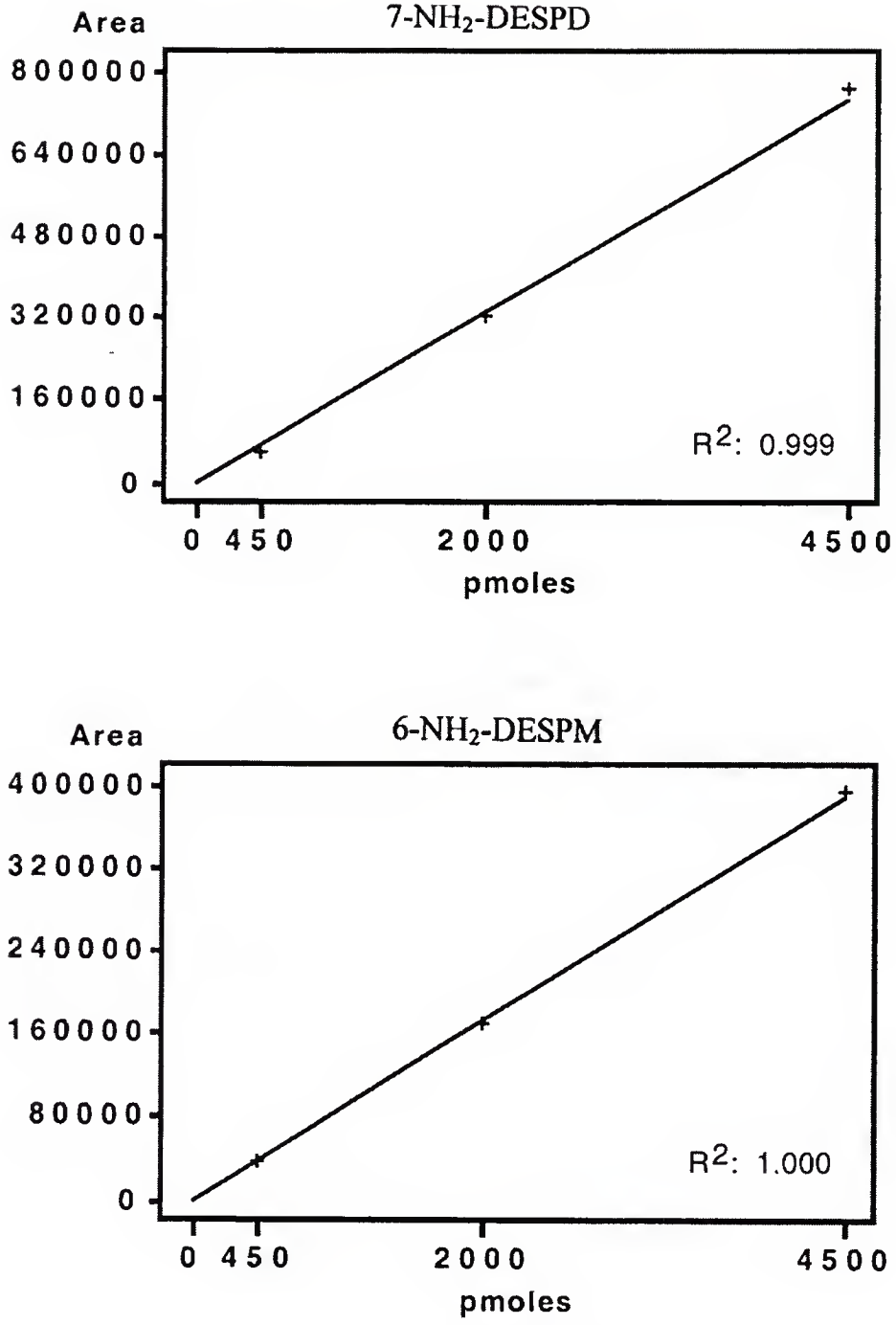
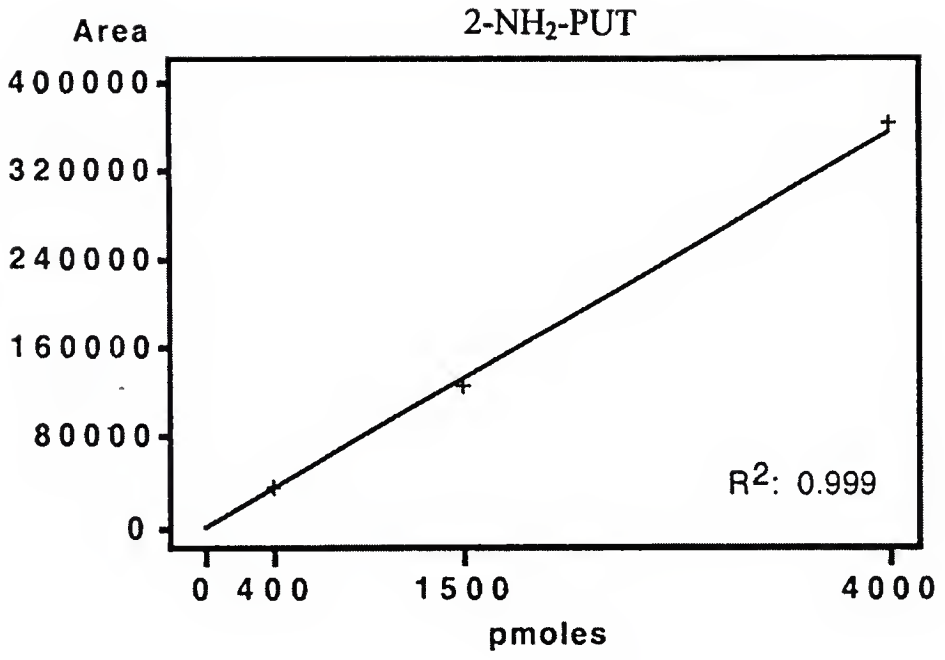


Figure 6-6. HPLC calibration curve for 7-NH_2-DESPD and 6-NH_2-DESPM. Each plot shows the amount (in picomoles) of each analogue versus the area under the curve.



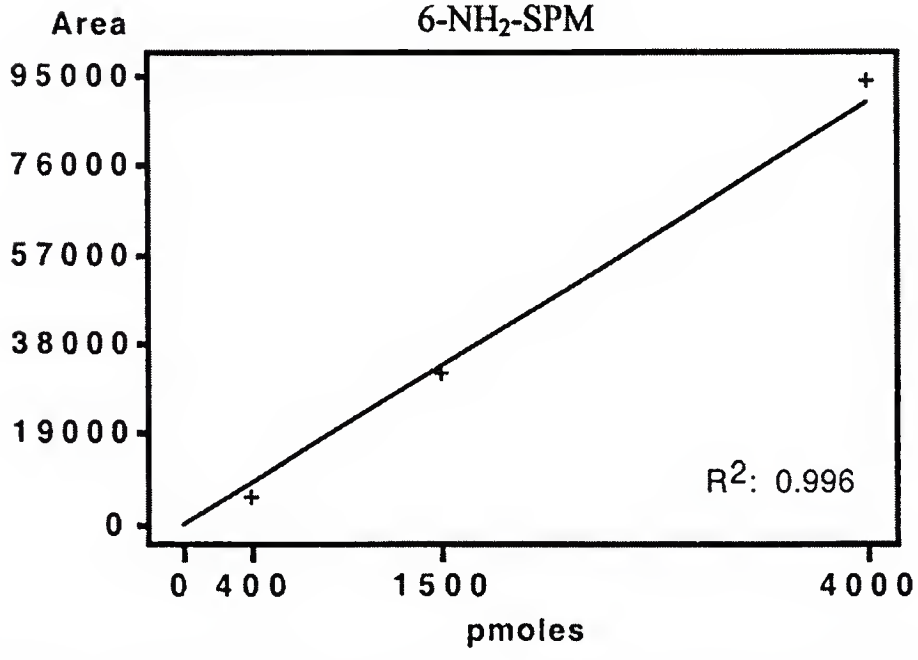


Figure 6-7. HPLC calibration curve for 2-NH_2-PUT and 6-NH_2-SPM. Each plot shows the amount (in picomoles) of each analogue versus the area under the curve.

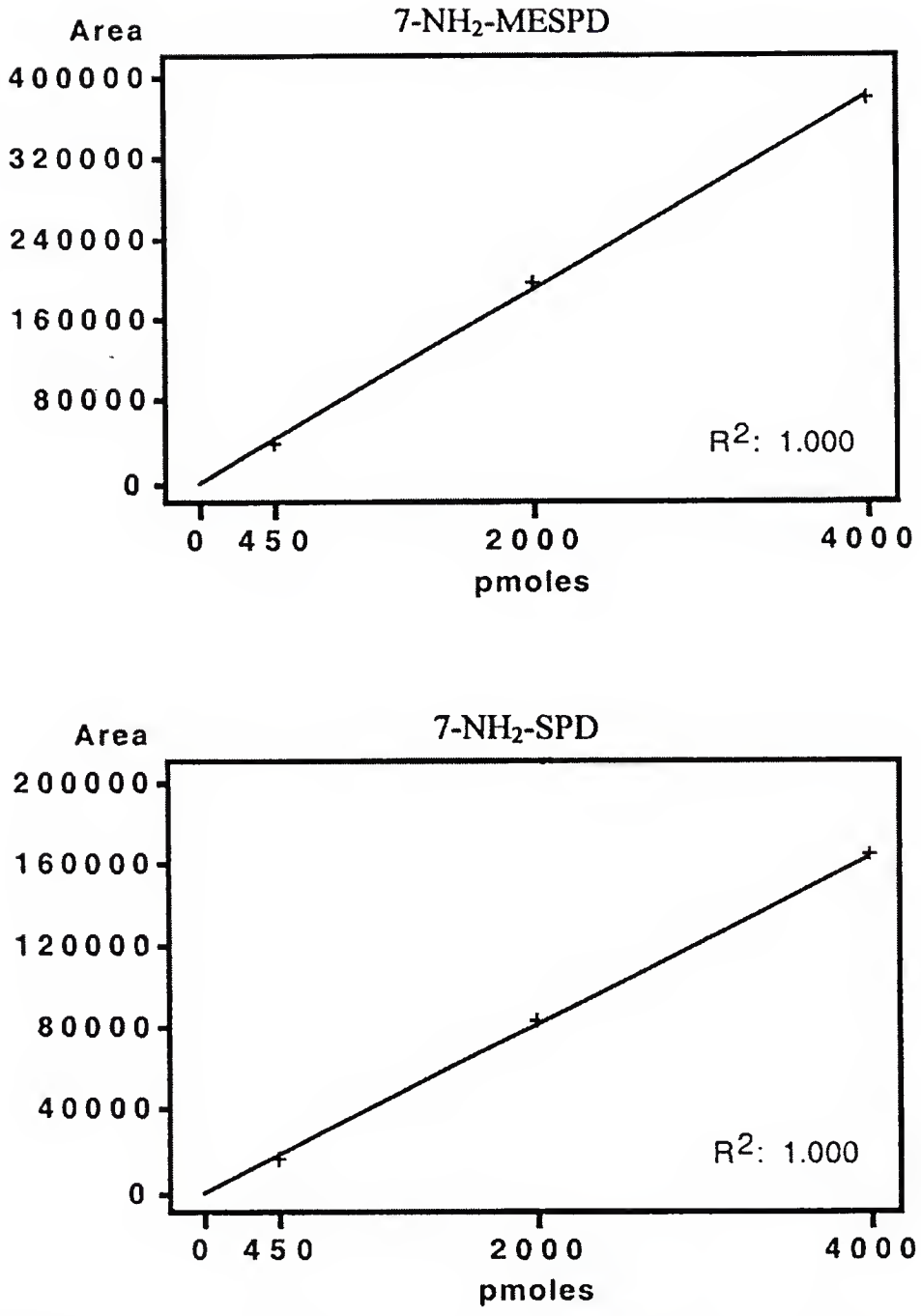
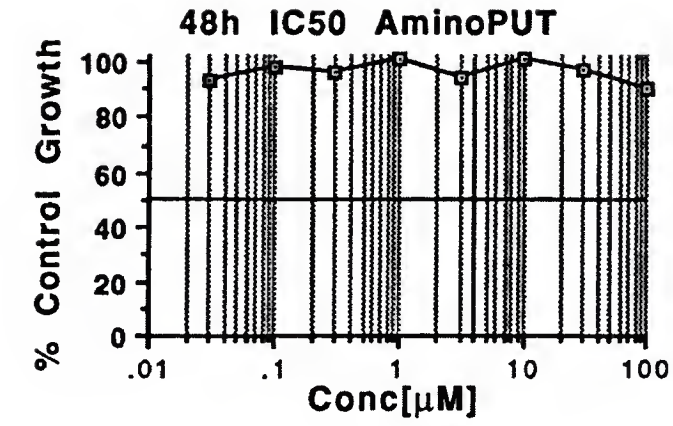


Figure 6-8. HPLC calibration curve for 7-NH_2-MESPD and 7-NH_2-SPD. Each plot shows the amount (in picomoles) of each analogue versus the area under the curve.



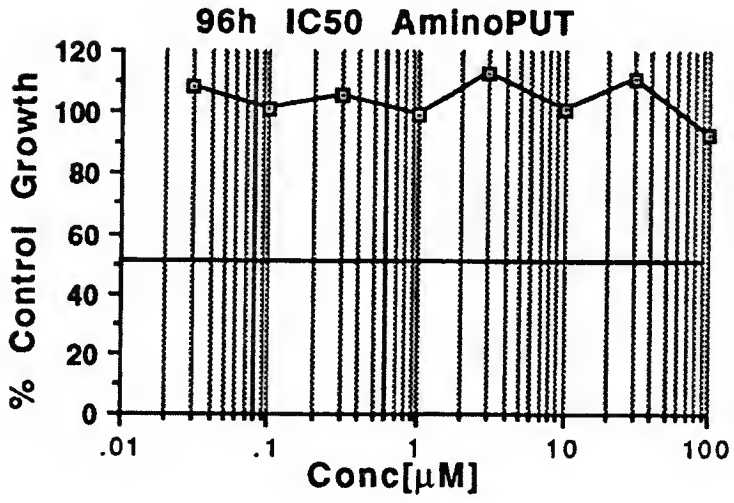


Figure 6-9. IC_{50} (48 and 96 h) graphs of 2-NH_2-PUT (aminoPUT). IC_{50} values were estimated at the point where the IC_{50} curve intersect with the 50% line.

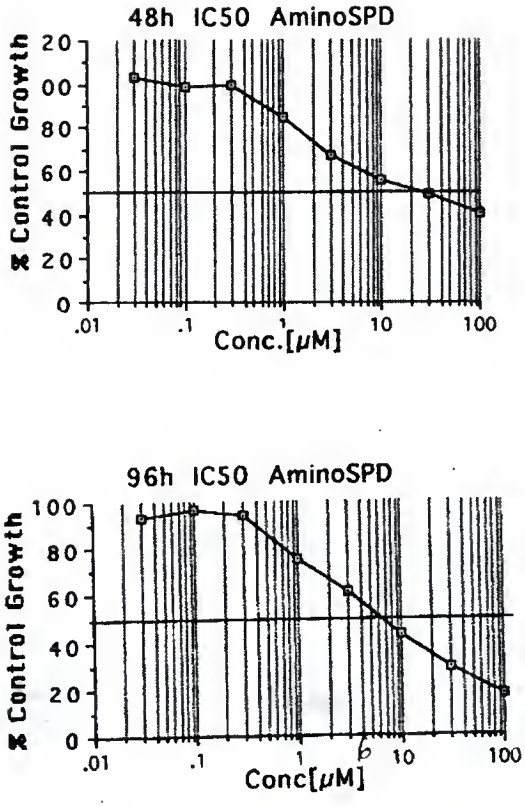


Figure 6-10. IC_{50} (48 and 96 h) graphs of 7-NH_2-SPD (aminoSPD). IC_{50} values were estimated at the point where the IC_{50} curve intersect with the 50% line.

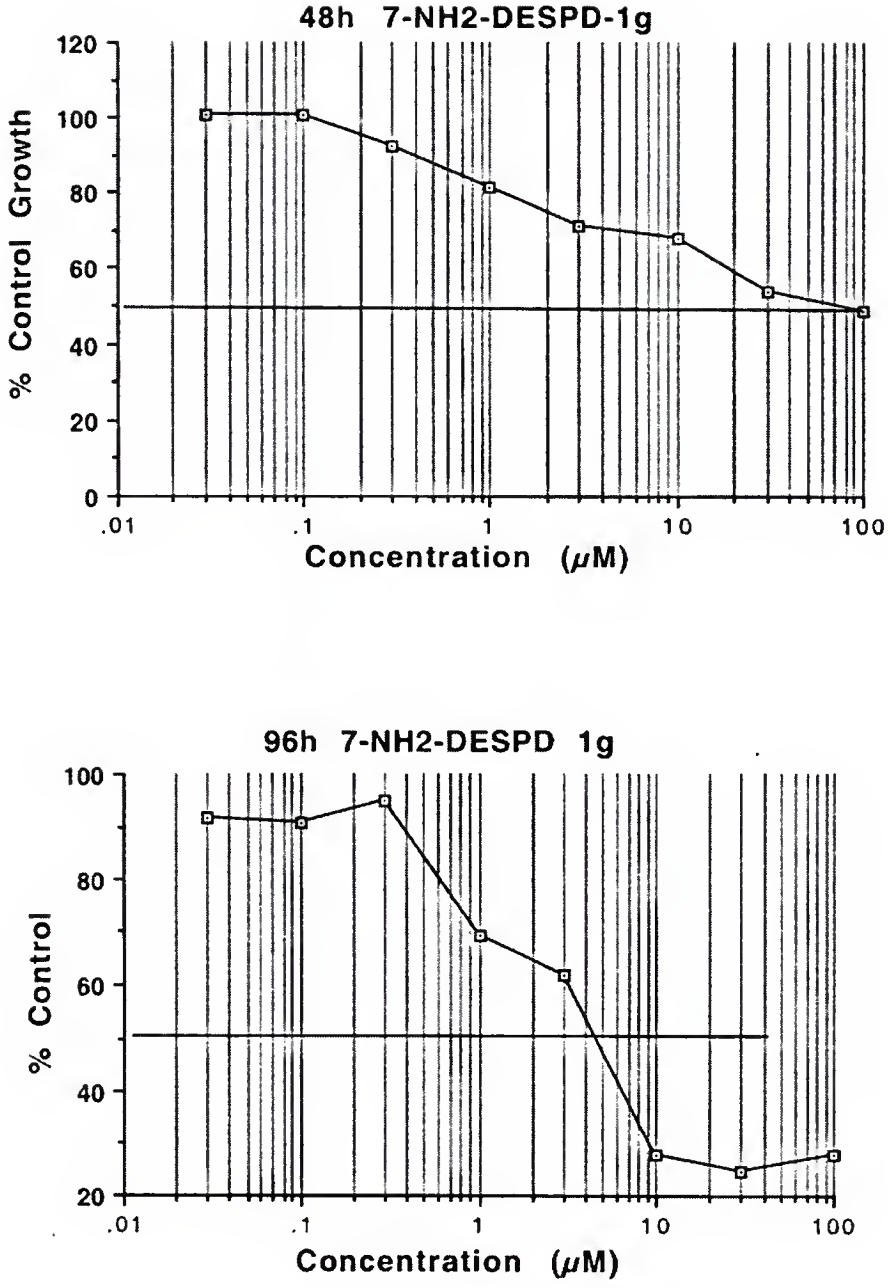


Figure 6-11. IC_{50} graphs (48 h and 96 h) of 7-NH_2-DESPD. IC_{50} values were estimated at the point where the IC_{50} curve intersects with the 50% line.

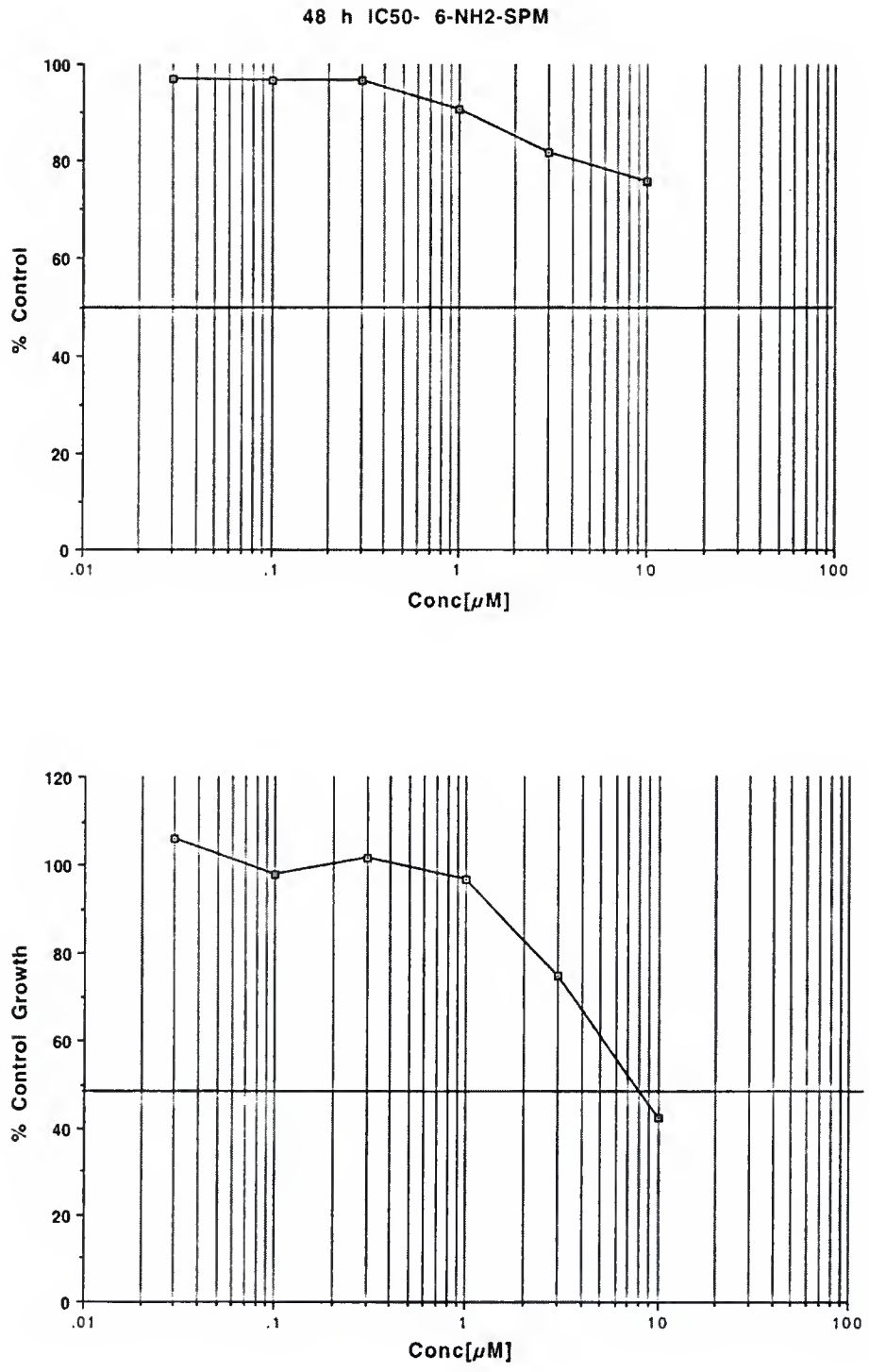


Figure 6-12. IC_{50} graphs (48 h, top; 96 h, bottom) of 6-NH_2-SPM. IC_{50} values were estimated at the point where the IC_{50} curve intersects with the 50% line.

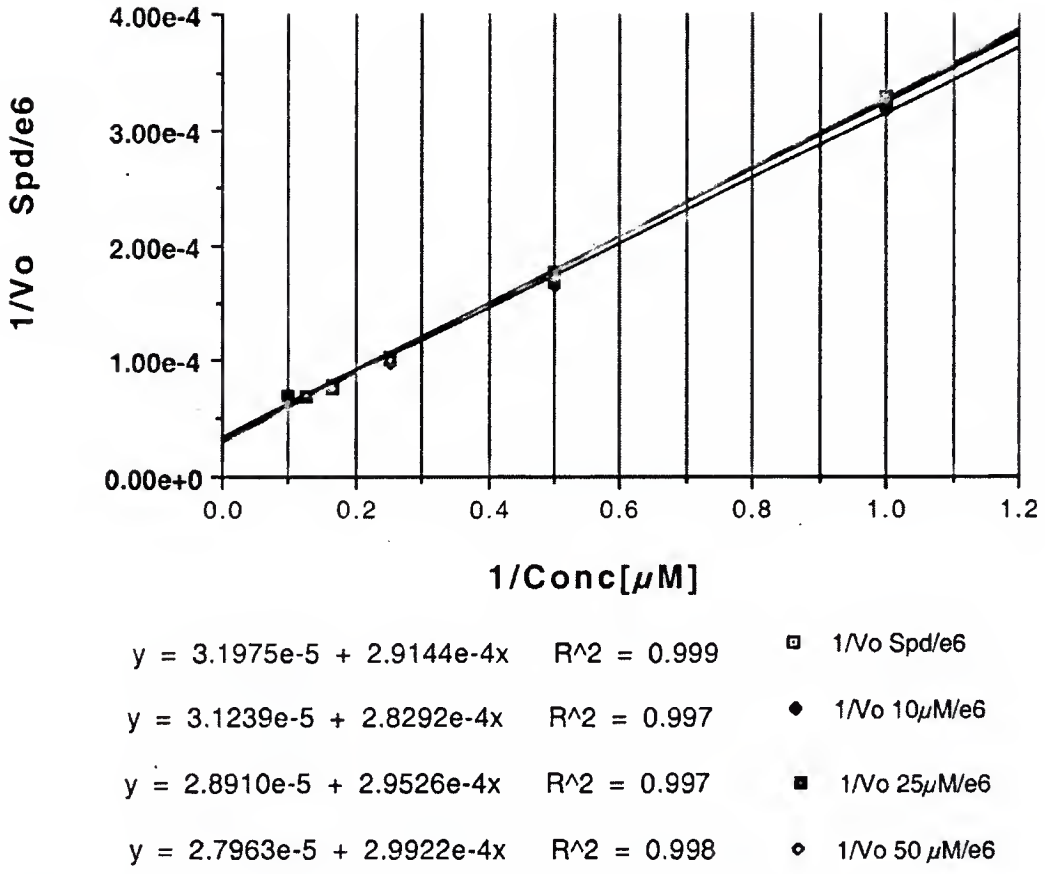


Figure 6-13. K_i graph of 2-NH_2-PUT. Each equation represents $K_{apparent}$ at the corresponding studied concentration. K_i was calculated based on the formula in chapter 6.

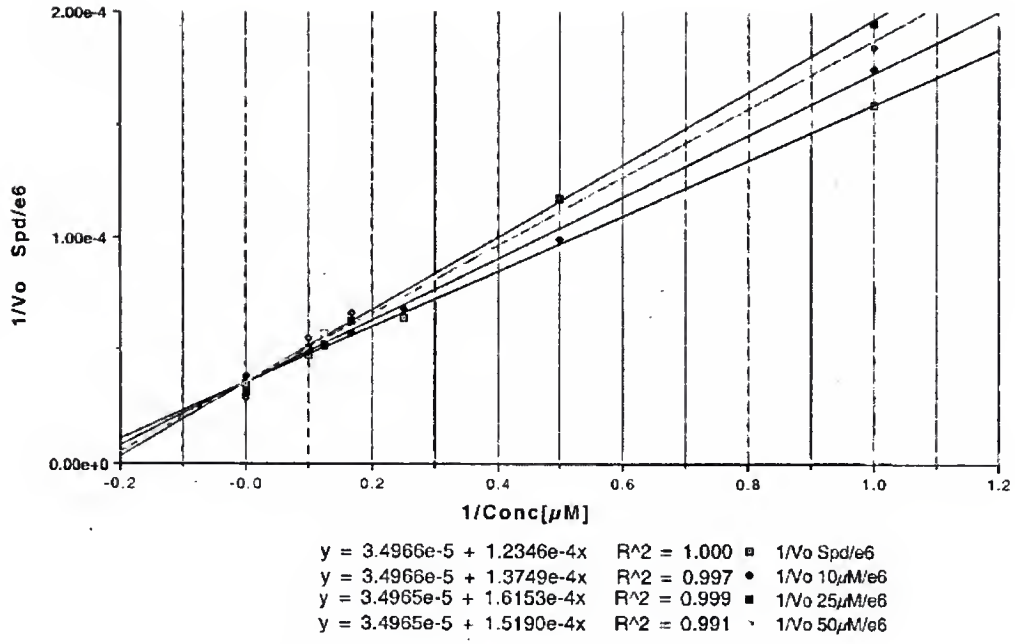


Figure 6-14. K_i graph of 7-NH_2-SPD. Each equation represents $K_{apparent}$ at the corresponding studied concentration. K_i was calculated based on the formula in chapter 6.

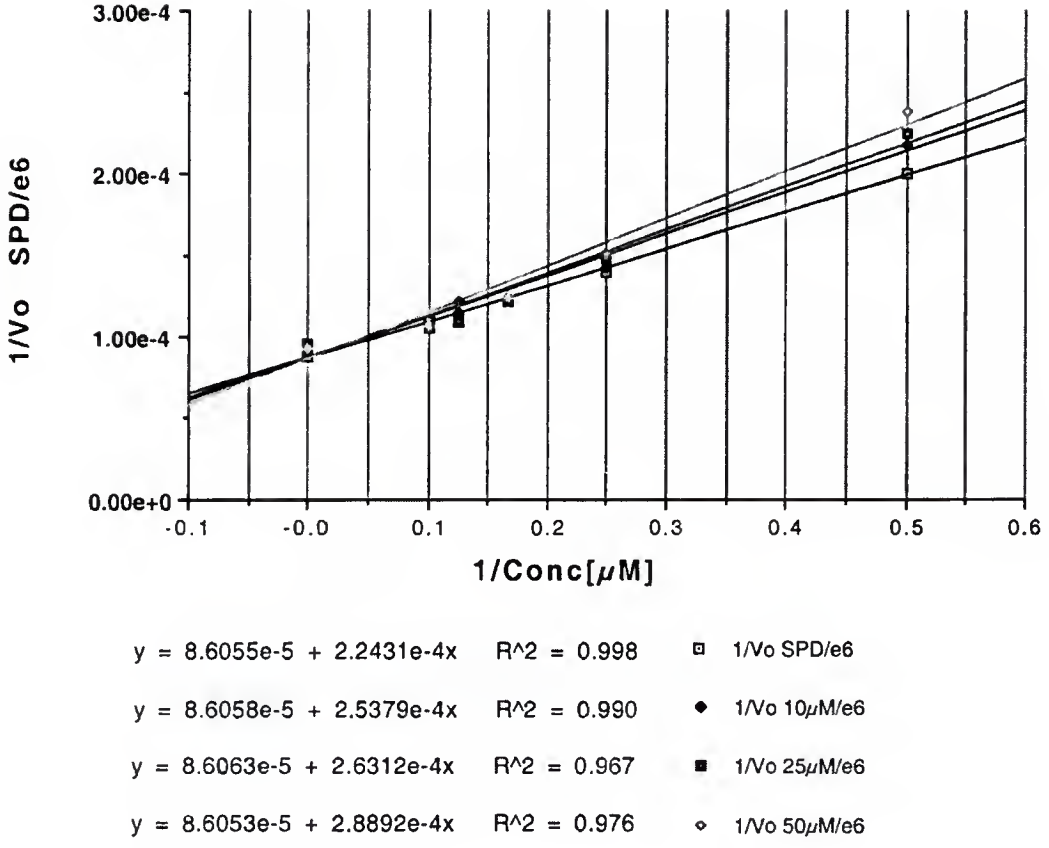


Figure 6-15. K_i graph of 7-NH_2-DESPD. Each equation represents $K_{apparent}$ at the corresponding studied concentration. K_i was calculated based on the formula in chapter 6.

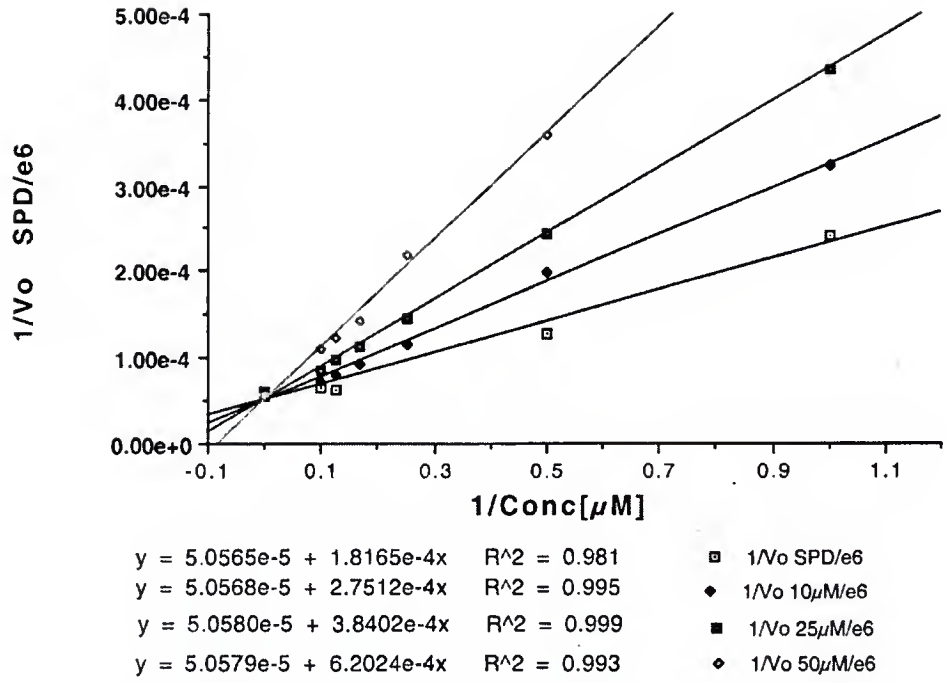


Figure 6-16. K_i graph of 6-NH_2-SPM. Each equation represents $K_{apparent}$ at the corresponding studied concentration. K_i was calculated based on the formula in chapter 6.

Table 6-1. HPLC method for polyamine analysis. Mobile phase A contains 93% aqueous methanol. Mobile phase B contains 65% aqueous acetonitrile.

Time (minute: second)	Percent of mobile phase A	Percent of mobile phase B
0:00	0.0	100.0
0:02	0.0	100.0
0:05	0.0	100.0
0:07	0.0	100.0
28:00	100.0	0.0
38:00	100.0	0.0
40:00	0.0	100.0
40:05	0.0	100.0
45:00	0.0	100.0

Table 6-2. Approximate retention times of natural polyamines and their analogues.

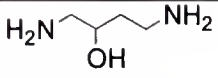
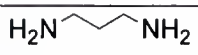
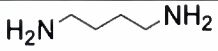
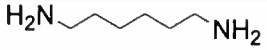
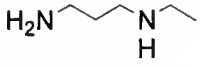
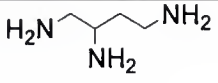
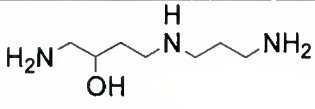
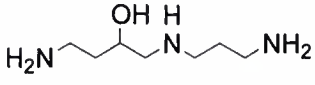
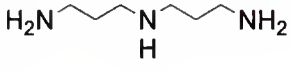
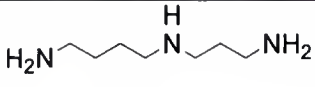
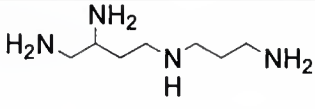
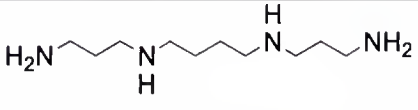
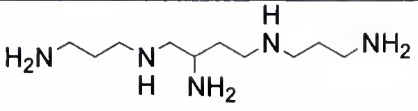
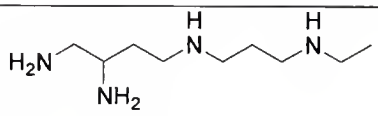
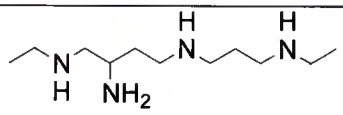
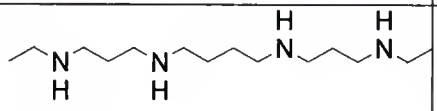
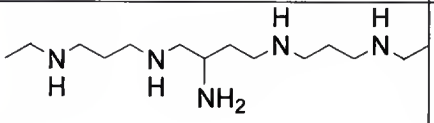
Polyamines/Analogues	Structures	Abbreviations	Retention time (min)
2-Hydroxyputrescine		2-OH-PUT	6.7
1,3-Diaminopropane		DAP	9.9
Putrescine		PUT	11.4
1,6-Diaminohexane		DHX	14.7
N-Ethyl-diaminopropane		MEDAP	15.7
2-Aminoputrescine		2-NH_2-PUT	18.9
7-Hydroxyspermidine		7-OH-SPD	19.5
6-Hydroxyspermidine		6-OH-SPD	20
Norspermidine		NSPD	21.4
Spermidine		SPD	22
7-Aminospermidine		7-NH_2-SPD	25.5
Spermine		SPM	27
6-Aminospermine		6-NH_2-SPM	29.8

Table 6-2. Continued

N^1-Ethyl-7-aminospermidine		7-NH_2-MESPD	28.1
N^1,N^8-Diethyl-7-aminospermidine		7-NH_2-DESPD	29.4
N^1,N^{12}-Diethylspermine		DESPM	31.4
N^1,N^{12}-Diethyl-6-aminospermine		6-NH_2-DESPM	33

CHAPTER 7
SUMMARY AND CONCLUSION

Synthesis of the racemic mixture of 2-NH_2-PUT was successful using the method of Altman with a slight modification. We extended this strategy to the synthesis of 7-NH_2-SPD. Histamine was first elaborated by the specific addition of an aminopropyl fragment to the N-4 position. This was achieved successfully based on our experience with alkylation of the terminal amino groups. The primary nitrogen was first protected by Mts, leaving this nitrogen with only one available acidic hydrogen. The resulting N-sulfonamide was converted to an anion by reaction with NaH in DMF. The mono-alkylation and subsequent removals of protecting groups provided N^4-aminopropyl-histamine. This histamine derivative was then utilized in a Bamberger ring cleavage reaction, and the corresponding intermediates were reduced to afford amino-protected 7-NH_2-SPD. After removals of all protecting groups, 7-NH_2-SPD trihydrochloride was synthesized in 10% overall yield.

We have developed a new method for the synthesis of 7-NH_2-DESPD. The most important, also the most interesting, part of this synthesis was the conversion of the hydroxyl group to an amine. The hydroxyl group was first converted to the corresponding *O*-tosyl, a good leaving group which was then displace by a phthalimide anion. Despite the weak nucleophilicity of the phthalimide anion and the steric hindrance of the electrophilic carbon, the displacement of the *O*-tosyl was achieved successfully in a surprising 90 % yield. The aminopolyamine 7-NH_2-DESPD was obtained in 9% overall yield.

Preparation of 6-NH_2-SPM was performed according to a method similar to that of the synthesis of 7-NH_2-DESPD. Selective elaboration of 4-bromo-1,2-epoxybutane followed by a specific hydrolysis of the epoxide ring provided a "fully protected" polyamine with a hydroxyl group at the C-6 position. The hydroxyl group was then converted to a good leaving group, *O*-tosyl, which was subsequently replaced by the N-phthalimide. Conversion from N-phthalimide to free amine was readily achieved by using $NaBH_4$ followed by acetic acid as described previously. The N-benzyl protecting groups in N^1,N^{12}-dibenzyl-6-aminospermine were cleanly removed by hydrogenation in acidic conditions to afford 6-NH_2-SPM. When attempting to perform this hydrogenation reaction at room temperature, incompletion of the removal of the benzyl group resulted. However, the same reaction was successfully achieved at 55 ^{0}C. Thus, temperature was the key to the hydrogenation reaction. The aminopolyamine 6-NH_2-SPM was generated in 5.5% overall yield.

In addition to the extension of our expertise in polyamine synthesis, this dissertation has provided us with additional insights into polyamine metabolism of synthetic polyamine analogues. In L1210 cells, a eukaryotic model, the biochemical machinery for metabolism of aminopolyamines was expressed. The key polyamine metabolic enzymes, ODC and AdoMetDC, were slightly affected by the aminopolyamine analogues. Upon exposure to 2-NH_2-PUT, L1210 cells synthesized 7-NH_2-SPD. Spermidine synthase catalyzed the transfer of an aminopropyl moiety from dcSAM to PUT. The resulting 7-NH_2-SPD was further aminopropylated selectively at the aminobutyl end by spermine synthase to form 6-NH_2-SPM. L1210 cells were also able to incorporate 7-NH_2-SPD and 6-NH_2-SPM into their polyamine catabolic network. When

exposed to 7-NH_2-SPD, the cell processed this polyamine analogue to form 2-NH_2-PUT. Similarly, 6-NH_2-SPM was taken up by the cells and converted to 7-NH_2-SPD, and then to 2-NH_2-PUT. Apparently, the catabolic cytosolic enzymes SSAT and the flavoprotein, PAO, must have been involved in the processing of aminopolyamines. SSAT transferred an acetyl group from acetyl coenzyme A to the terminal amino of the aminopropyl fragment of 7-NH_2-SPD and 6-NH_2-SPM. The corresponding intermediates N^1-acetyl-7-NH_2-SPD and N^1-acetyl-6-NH_2-SPM were then oxidized by PAO, and the resulting imines were hydrolyzed to 2-NH_2-PUT and 7-NH_2-SPD, respectively. In both of these catabolic instances, 3-acetamidopropanal was also produced. This aldehyde is known to be further oxidized to *N*-acetyl-β-alanine, which is then deacetylated to β-alanine. Thus, the putrescine moiety is conserved in the polyamine metabolic cycle, whereas the aminopropyl moieties originating from methionine are irreversibly eliminated as β-alanine. We also have reported that, in addition to SSAT/PAO pathway, N^8-deethylation was a critical pathway in the catabolism of 7-NH_2-DESPD, while N^1-deethylation was a crucial pathway in the catabolism of 6-NH_2-DESPM. Small quantities of each of the expected aminopolyamine metabolites (N^1-ethyl-7-aminospermidine and 7-aminospermidine) were detected in the intracellular polyamine pool of L1210 cells.

In *Pseudomonas aeruginosa*, similar biosynthetic and catabolic activities operated on aminopolyamines. Elaboration from 2-NH_2-PUT to 7-NH_2-SPD was observed. However, 6-NH_2-SPM was not biosynthesized from 7-NH_2-SPD inside the bacteria. This observation was consistent with results of many other studies that *P. aeruginosa* does not contain spermine synthase, the enzyme responsible for SPM synthesis. Similar to L1210 cells, these bacteria were able to convert 6-NH_2-SPM to 7-NH_2-SPD and then to

2-NH_2-PUT. It was interesting that the bacteria were not able to biosynthesize 6-NH_2-SPM from the respective substrate, but they were able to process this exogenous polyamine analogue. We also reported the metabolic processing of 7-NH_2-DESPD and 6-NH_2-DESPM in *P. aeruginosa*. Expected aminopolyamine metabolites (N^1-ethyl-7-aminospermidine, 7-aminospermidine and 2-aminoputrescine) were detected in significant quantities.

Understanding the incorporating and processing of various aminopolyamines in cells and bacteria has provided us with additional knowledge on polyamine metabolism. The polyamine metabolic network has been recognized as a potential target for therapeutic intervention. This dissertation provides additional insights into polyamine metabolism of eukaryotes and prokaryotes, which may help to develop polyamine analogues with potential anticancer and antibacterial activities.

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BIOGRAPHICAL SKETCH

John van Nguyen (also known as Nhut Nguyen) was born in South Vietnam on October 27, 1973. He graduated from Long Xuyen I High School, Vietnam, in 1990. Sponsored by the U.S. government, he and his family migrated to the U.S.A. in November 1991. After attending Los Angeles Pierce College, Woodland Hills, California, from 1992 to 1995, he was admitted to the University of California at Riverside, California, where he received his B.S. degree in chemistry with honors, in June of 1998. John Nguyen was then accepted to the Medicinal Chemistry Department at the University of Florida, where he began his graduate studies in August 1998, under the supervision of Dr. Raymond Bergeron. He was awarded the College of Pharmacy Dean's Scholarship. John Nguyen received a Ph.D. degree in medicinal chemistry in December 2004.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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